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(54) Title: CYSTEINE–DEPLETED PEPTIDES RECOGNIZED BY A3–RESTRICTED CYTOTOXIC LYMPHOCYTES, AND USES THEREFOR

(57) Abstract

Cysteine–depleted CTL epitopes can elicit a stronger or more specific CTL response than the native, cysteine–containing CTL epitope of a disease associated antigen.

CYSTEINE-DEPLETED PEPTIDES RECOGNIZED BY
A3-RESTRICTED CYTOTOXIC LYMPHOCYTES, AND USES THEREFOR

This application claims priority under §119(e) from
U.S.S.N. 60/037,781, filed January 30, 1997, hereby incorporated
5 by reference.

CROSS-REFERENCE TO RELATED APPLICATIONS

Ser. No. 60/027,627, filed October 4, 1996, now pending,
Ser. No. 60/013,972, filed March 19, 1996, now pending,
PCT/US95/01991, filed February 16, 1995, Ser. No. 08/234,784,
10 filed April 29, 1994, now pending, Ser. No. 08/197,399 filed
February 16, 1994, now pending, are all hereby incorporated
by reference in their entirety.

Mention of Government Grant

Certain aspects of the invention may have been supported
15 by NIH grants CA57653, AI21393, AI20963 and AI 33993. The
U.S. Government may have certain rights in the invention.

BACKGROUND OF THE INVENTION
Field of the Invention

The present invention is directed to peptides that, in
20 association with Class I MHC molecules, form epitopes
recognized by cytotoxic T-cells specific for human diseases,
to immunogens comprising said epitopic peptides, and to
related compositions, methods and apparatus.

Description of the Background Art

25   There are two basic immune defenses in vertebrates.
Humoral immunity is provided by antibodies, which defend
primarily against bacterial, and the extracellular phase of
viral, infections. Cellular immunity is provided by T cells,
and their response is effective against microorganisms,
30 parasites, cancer cells, foreign tissue (transplants), and
both the intracellular and extracellular phase of viral
infections. The cytotoxic T cell (lymphocyte) is a
particular class of cellular immune response effector cell
which eliminates invaders directly, e.g., by lysis. The two

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systems of cellular immunity interact in complex ways.

The present invention therefore contemplates the inhibition of diseases caused by viruses, bacteria, fungi, protozoa, algai, and other microorganisms, by multicellular
5 parasites, and by cancer cells.

The prevention and treatment of cancer, and especially of melanoma, is of particular interest.

Melanoma affects 30,000 new patients per year in the United States. It is a cancer manifested by the unabated
10 proliferation of melanocytes. Eighty percent of melanoma patients are diagnosed during their productive years between the ages of 25 and 65. The incidence of melanoma is rapidly increasing, in 1935 the lifetime risk of developing melanoma was 1:1,500 individuals, at present, the risk has risen to
15 1:105. It is believed that by the year 2000 the risk of developing melanoma will increase to about 1:70 to 1:90. Early diagnosis and treatment of this disease is crucial. Once a primary tumor becomes metastatic the disease is almost always fatal.

20   Cytotoxic lymphocyte (CTL) response has been shown to be an important host defense against malignant cells, Rock et al. J. Immunol., (1993), 150:1244.

Lymphocytes isolated from patients having melanoma, when stimulated in vitro with recombinant interleukin-2 (rIL-2)
25 and autologous melanoma cells, develop a melanoma specific cytotoxic response, Vose et al., Nature, (1982), 296:359; Knuth et al., Proc. Natl. Acad. USA, (1984), 81:3511; Slingluff et al., Arch. Surg., (1987), 122:1407; Darrow et al., Cancer, (1988), 62:84; Slingluff et al., J. Natl. Cancer
30 Inst., (1988), 80:1016; Slingluff et al., Ann. Surg., (1989), 210:194; Muul et al., J. Immunol., (1987), 138:989; Van den Eynde et al., Int. J. Cancer, (1989), 44:634; Anichini et al., Int. J. Cancer, (1985), 35:683. The majority of melanoma-specific effector lymphocytes are CD8^{+} cytotoxic T
35 lymphocytes (CTL) that are restricted by class I Major Histocompatibility Complex (MHC) molecules, Vose et al; Slingluff et al (1988), supra, Hersey et al., Cancer Immunol. Immunother., (1986), 22:15. These characteristics are

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present whether CTL have been generated from peripheral blood
lymphocytes (PBL), lymph node cells, or tumor infiltrating
lymphocytes.

    The evidence that the CTL response to human melanoma is
5 restricted by class I MHC molecules includes demonstration of
cross-reactivity for allogenic melanoma cells that share a
restricting class I MHC molecule with the autologous tumor.
The HLA-A2 molecule and its variants, of which HLA-A2.1 is by
far the most common, is an effective restricting element for
10 the melanoma-specific CTL response. Additionally, melanoma-
specific HLA-restricted CTL lyse the majority of A2⁺ melanomas
tested, Darrow et al., J. Immunol., (1989), 142:3329; Wolfel
et al., J. Exp. Med., (1989), 170:797; Hom et al., J.
Immunother., (1991), 3:153. By demonstrating lysis of A2-
15 melanomas transfected with the A2.1 gene, it has been shown
that these transfected melanomas can present the epitopes
recognized by A2-restricted melanoma-specific CTL, Kawakami
et al., J. Immunol., (1992), 148:638. These results suggest
that these CTL recognize A2-restricted epitopes that are
20 shared by the majority of melanomas, although very little is
known about the number and identity of their epitopes.

    Class I molecules of the Major Histocompatibility
Complex (MHC) bind to peptides derived from intracellular
pathogens or from proteins expressed in tumor cells, and
25 present them on the cell surface to the host immune system.
The mechanism of peptide presentation involves protein
synthesis and proteolysis in the cytosol, followed by
transport of peptides into the endoplasmic reticulum (ER),
through the action of the TAP transporter molecules.
30 Peptides then become associated with newly synthesized class
1 molecules, and the resulting complexes move to the cell
surface. Proteins that are membrane associated or secreted
contain signal sequences that cause them to be contransla-
tionally transferred into the ER from membrane-bound
35 ribosomes. Such proteins would thus be protected from the
action of cytoplasmic proteases. However, since peptide
epitopes do arise from such proteins, although their TAP
dependent expression is unclear, it has been assumed that the

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proteolysis to generate these peptide epitopes occurs after these proteins have been aberrantly translated on cytoplasmic ribosomes.

5   Adoptive transfer of tumor stimulated CTL has been as-
sociated with some tumor regressions, Rosenberg et al., N. Eng. J. Med., (1988), 319:1676.

An alternate approach to augmenting the T-cell response to melanoma is the use of a vaccine to stimulate CTL in vivo (active specific immunotherapy). Epitopes for CD8⁺ CTL are

10   believed to be short, usually 9- residue peptides that bind to a cleft on the surface of the class I MHC molecule, Udaka et al., Cell, (1992), 69:989; VanBleek et al., Nature, (1990), 348:213; Falk et al., J. Exp. Med., (1991), 174:425. These peptides, generated from proteolysis of endogenous

15   proteins in the cytosol, are transported to the endoplasmic reticulum, where they become associated with newly syn-
thesized class I MHC molecules. They are then transported to the cell surface, Elliott et al., Nature, (1990), 3348:195. CTL epitopes have been reconstituted in vitro by allowing

20   exogenous peptides to bind to MHC molecules on the cell surface of target cells, Townsend et al., Annu. Rev. Immunol., (1989), 7:601.

Several CTL epitopes have been identified which comprise cysteine, e.g., MLLAYLYCL (see Table A and Boon, et al., Eur.

25   J. Immunol., 24:759-64 (1994)). However, prior to the priority application, it had not been suggested that it could be advantageous to replace these cysteines with non-cysteine residues. Indeed, there would have been concern that such replacement would adversely effect the interaction of the

30   epitope with the class I MHC molecule or with the T cell receptor, or the specificity of the response to the epitope.

Meadows, et al., Immunity, 6:273-81 (1997), published after the priority date, discloses a nine residue H-Y specific CTL epitope FIDSYICQV. It was discovered that

35   cysteinylation of the cysteine affected recognition, improving recognition by one clone, and reducing recognition by another.

DiModugno, et al, J. Immunother., 20:431-6 (1997),

published after the priority date, characterized certain
cysteine-containing CTL epitopes (HLYQCQVV and CLTSTVLV) of
the HER-2/neu gene product. DiModugno made Ser and Ala
substitution mutants of CLTSTVLV to prevent dimenzation;
5 there was no effect, good or bad, on the binding of the
peptide to high affinity HLA-A2.1 molecules, and hence no
motivation to perform such substitutions on other epitopes in
the future.

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SUMMARY OF THE INVENTION

The present invention is particularly directed to "cysteine-depleted" CTL epitopes, in which one or more cysteines not essential to eliciting the desired CTL response

5   are replaced by another amino acid, especially Ala, Thr, Ser or Gly.

The present invention relates to immunogens which are capable of eliciting a disease-specific cytotoxic lymphocyte response in at least some individuals, which response is

10 directed to peptide epitopes carried by those immunogens, and to the use of those immunogens in active specific immunotherapy and immunoprophylaxis against that disease. Melanoma is of particular interest.

These immunogens may be used as vaccines, in active

15 specific immunotherapy. The immunogens may be administered directly or by gene therapy. The epitopic peptides may also be used to stimulate lymphocytes, the latter then being used for adoptive immunotherapy.

An antigen associated with a particular disease may

20 elicit a CTL response, in which case the antigen must be processed into peptide fragments which are CTL epitopes. In some instances these native CTL epitope contains cysteine. If so, it is possible that intermolecular disulfide bonds will form between cysteines of two different epitopic

25 peptides. If the epitope contains two or more cysteines, it is possible to form intramolecular disulfide bonds, too. Applicants have discovered that such disulfide bond formation can interfere with the CTL response.

One may reduce the total Cys to one, thus preventing

30 intramolecular disulfide bond formation, or to zero, thereby also blocking intermolecular disulfide bonding.

In a preferred embodiment, the native CTL epitope is one other than DiModugno's CLTSTVQLV, which is a CTL epitope of a breast and ovarian tumor-associated antigen.

35   In other preferred embodiments, the cysteine depletion is applied to a cysteine-incorporating CTL epitope of the melanoma-associated antigens pMel-17 or tyrosinase.

A native tyrosinase CTL epitope of particular interest

is the peptide Lys-Cys-Asp-Ile-Cys-Thr-Asp-Glu-Tyr (SEQ ID NO:93). We have discovered that substitution of Ser or Ala for Cys-2 in peptide KCDICTDEY increases the ability to reconstitute the epitope in vitro, probably by prevention of

5 intramolecular disulfide bonds. The same principles apply to the related EKCDICTDEY and DAEKCDICTDEY sequences, and indeed to any Cys-containing CTL epitopes.

Additional embodiments of the present invention are described below. References to melanoma should be deemed to

10 apply, _mutatis mutandis_, to other diseases, too.

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BRIEF DESCRIPTION OF THE DRAWINGS

   Figures 1A-B. Recognition of autologous and HLA-matched
melanomas by melanoma-reactive CTL. In 1A), VMM12 CTL are
evaluated for lysis of a panel of target cells. The VMM12
5   CTL recognize shared melanoma antigens presented by HLA-A1
(VMM15 melanoma cells share HLA-A1 with VMM12), and by HLA-A3
(VMM10 melanoma cells share HLA-A3 with VMM12). Similarly,
in 1B), VMM15 CTL are evaluated in the same manner. VMM15
CTL recognize shared melanoma antigens presented by HLA-A1
10 (VMM12 melanoma cells) and by either HLA-A1, -A25, or -B8
(VMM14 melanoma cells).

   Figures 2A-B. HLA-A1+ CTL lines recognize tyrosinase
peptides on HLA-A1. In 2A), VMM12 CTL are capable of lysing
C1R-A1 cells infected with a vaccinia-tyrosinase construct.
15 In 2B), VMM15 CTL also recognize tyrosinase.

   Figure 3. VMM15 CTL recognize peptides containing
KCDICTDEY in association with HLA-A1. C1R-A1 cells were
pulsed with 10 uM, 1 uM and 0.1 uM concentrations of
synthetic peptides prior to addition of VMM15 CTL.
20 Background lysis of C1R was approximately 10%. Direct
cytotoxicity by the peptides themselves was negligible (open
diamonds), averaging 0-2%. An epitope for VMM15 CTL was
reconstituted by three of the test peptides, numbers 5, 12,
and 15, corresponding to KCDICTDEY, DAEKCDICTDEY, and
25 EKCDICTDEY as marked.

   Figure 4. VMM12 CTL recognize a peptide containing
KCDICTDEY in association with HLA-A1. C1R-A1 cells were
pulsed with peptides at 1 to 0.01 uM concentrations prior to
adding VMM12 CTL. The peptides themselves were not cytolytic
30 (open diamonds). The peptide DAEKCDICTDEY reconstituted an
epitope for these VMM12 CTL, although weakly.

   Figure 5. Amino acid sequence of tyrosinase, with the
position of KCDICTDEY highlighted and underlined. The high
proportion of cysteine residues and acidic residues are noted
35 relative to the proportion in the intact protein.

   Figure 6. Synthetic peptides from the tyrosinase
protein were tested for recognition by HLA-A1-restricted

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<u>VMM15 CTL.</u> C1R cells were labeled with ^{51}Cr and pulsed with the indicated crude synthetic peptides at approximately 20 micrograms/ml for 2 hours prior to CTL co-culture. CTL were used at an E:T of 15:1. Open bars indicate lysis in the

5 presence of CTL. As a control for possible direct peptide toxicity, lysis was also measured in the absence of CTL (filled bars). Reconstitution of the CTL epitope was observed for three peptides: KCDICTDEY, EKCDICTDEY, and DAEKCDICTDEY, with 25-40% target cell lysis for those three

10 peptides, and with 10% background lysis of target cells pulsed with irrelevant peptides.

  <u>Figure 7</u>. <u>VMM15 CTL recognize, at comparable doses, 9-,</u> <u>10- and 12- residue peptides containing the amino acid</u> <u>sequence KCDICTDEY in association with HLA-A1.</u> These three

15 synthetic peptides were purified by HPLC, and evaluated in a dose-titration assay. C1R transfectants expressing HLA-A1 (C1R-A1) were labeled with ^{51}Cr and then pulsed with the indicated peptides at various concentrations (abcissa) for 2 hours prior to CTL co-culture. Cytotoxicity was measured in a

20 stardard chromium release assay. Recognition of C1R-A1 cells pulsed with each of these KCDICTDEY-containing peptides was similar and, at the higher concentrations, lysis of these peptide pulsed targets equalled lysis of the autologous VMM15 melanoma cells. TPMFNDINIY is an irrelevant peptide derived

25 from tyrosinase that also fits the HLA-A1 binding motif. Lysis of non-peptide pulsed C1R cells was 19%, and lysis of VMM15 tumor cells (positive control) was 50%. An effector:target ratio of 25:1 was used.

  <u>Figure 8: Alanine or serine substitution at the more</u>

30 <u>amino-terminal cysteine greatly enhances VMM15 CTL</u> <u>recognition.</u> CTL recognition of alanine and serine substitued peptides. C1R transfectants expressing HLA-A1 (C1R-A1) were labeled with ^{51}Cr and then pulsed with the indicated peptides at various concentrations (abcissa) for 2

35 hours prior to CTL co-culture, at which time the peptide concentration dropped 2-fold. TPMFNDINIY is derived from tyrosinase and fits the HLA-A1 binding motif. Cytotoxicity was measured in a stardard chromium release assay. In **A**,

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VMM15 CTL were used at an E:T of 25:1. Lysis of autologous
tumor was 44%, lysis of non-peptide pulsed C1R-A1 was 3%. In
B, VMM15 CTL were used at an E:T of 10:1. Lysis of autologous
tumor was 70%, and lysis of non-peptide pulsed C1R-A1 was 4%.

5   **Figure 9. The dodecamer peptide DAEKSDICTDEY competes
with the nonamer peptide KSDICTDEY for recognition by VMM15
CTL.** B-LCL derived from patient VMM15 (VMM15 EBV) were ^{51}Cr-
labeled, pulsed with the peptide KSDICTDEY at a final
concentration of 20 μg/ml for 1 hour, then washed. Unlabeled
10   (cold) VMM15 EBV were similarly treated with KSDICTDEY,
DAEKSDICTDEY, or no peptide, as indicated. Increasing numbers
of these various unlabeled targets were pre-incubated with
VMM15 CTL for 1 hour at 37°C. Labeled (hot) targets were then
added for an additional 4 hour co-culture at a final
15   effector-to-labeled target ratio of 10:1.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS
OF THE INVENTION

The present invention relates to certain melanoma-specific CTL epitopes, and their incorporation into

5   immunogens for immunoprophylactic and immunotherapeutic purposes.

CTL Epitopes

An epitope may be said to be associated with a particular infectious disease if it is presented by an

10   intracellular, surface, or secreted antigen of the organism which causes the disease, or in the case of a virus, if it is associated with viral particles or is specific to a cell infected by the virus.

It may be said to be associated with a particular tumor

15   if it is presented by an intracellular, surface or secreted antigen of said tumor. It need not be presented by all cell lines of the tumor type in question, or by all cells of a particular tumor, or throughout the entire life of the tumor. It need not be specific to the tumor in question. An epitope

20   may be said to be "tumor associated" in general if it is so associated with any tumor.

The term "disease associated epitope" also includes any non-naturally occurring epitope which is sufficiently similar to an epitope naturally associated with the disease in

25   question so that cytotoxic lymphocytes which recognize the natural disease epitope also recognize the similar non-natural epitope.

An epitope may be said to be specific to a particular source (such as a disease-causing organism or a tumor), if it

30   is associated more frequently with that source than with other sources. Absolute specificity is not required, provided that a useful prophylactic, therapeutic or diagnostic effect is still obtained.

In the case of a "tumor-specific" epitope, it more

35   frequently associated with that tumor that with other tumors, or with normal cells. Preferably, there should be a statistically significant (p=0.05) difference between its

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frequency of occurrence in association with the tumor in
question, and its frequency of occurrence in association with
(a) normal cells of the type from which the tumor is derived,
and (b) at least one other type of tumor. An epitope may be
5   said to be "tumor-specific" in general is it is associated
more frequently with tumors (of any or all types) than with
normal cells. It need not be associated with all tumors.

The term "tumor specific epitope" also includes any non-
naturally occurring epitope which is sufficiently similar to
10 a naturally occurring epitope specific to the tumor in
question (or as appropriate, specific to tumors in general)
so that cytotoxic lymphocytes stimulated by the similar
epitope will be essentially as specific as CTLs stimulated by
the natural epitope.

15   In general, tumor-versus-normal specificity is more
important than tumor-versus-tumor specificity as (depending
on the route of administration and the particular normal
tissue affected), higher specificity generally leads to fewer
adverse effects. Tumor-versus-tumor specificity is more
20 important in diagnostic as opposed to therapeutic uses.

The reference to an epitope as being "restricted" by a
particular allele of MHC, such as HLA-A1, indicates that such
epitope is bound and presented by the allelic form in
question. It does not mean that siad epitope might not also
25 be bound and presented by a different allelic form of MHC,
such as HLA-A2, HLA-A3, HLA-B7, or HLA-B44.

The disease-specific CTL epitopes of the present
invention are peptides, typically 9-13 amino acids in length,
which are sufficiently similar to a disease-specific epitope
30 recognized by a disease-specific CTL to be useful, under
suitable conditions of use, to protect an individual from
that disease, or to be useful in the diagnosis of the disease
or of a patient's ability to fight the disease by a CTL
response. Preferably, these epitopes are substantially
35 identical with disease CTL peptide epitopes of naturally
occurring antigens associated with the disease recognized by
disease-specific CTLs. For the purpose of the present
invention, a melanoma-specific CTL epitope is an epitope

which is recognized by a T-cell receptor of at least some
cytotoxic lymphocytes of at least some individuals in the
population of interest, and which is more frequently or
strongly associated with melanoma cells than with at least
5   some other cancer and/or normal cells. There may be some
cross-reactivity, for example, with other cells of
melanocytic lineage. Absolute specificity is not required,
provided that a useful prophy-lactic, therapeutic or
diagnostic effect is still obtained.

10   A cysteine-depleted epitope is one which differs from a
native CTL epitope in that at least one cysteine residue of
the native epitope is replaced by a non-cysteine residue. By
definition, a cysteine-depleted epitope is not identical to a
native CTL epitope. However, a cysteine-depleted epitope
15 could be used in conjunction with the corresponding native
epitope, with a different native CTL epitope which does not
contain cysteine, a different native CTL epitope which does
contain cysteine, with a non-native CTL epitope which may or
may not contain cysteine, or with another cysteine-depleted
20 form of the same or a different native CTL epitope.

Melanoma-specific CTL epitopes which are identical to or
otherwise substantially homologous with a pMel-17 or
tyrosinase CTL eptiope are of particular interest. The
cysteine-depleted mutant of (DAE) KCDICTDEY (A1-restricted)
25 could be used by itself or in conjunction with other
epitopes, such as the pMel-17 epitopes YLEPGPVTA (Peptide
946L) (A2.1-restricted) and ALLAVGATK (A3-restricted), and
the tyrosinase epitope YMDGTNSQV (peptide 1030) (A2.1-
restricted). The "(DAE)" indicates optionally included
30 residues (or equivalents).

The family of melanoma epitopes which are recoverable
from an individual is dependent on the nature of the binding
site of the Class I MHC (HLA) molecules expressed by the
individual, and, as a result of the polymorphism of the Class
35 I MHC (HLA) molecules, can vary considerably from one
individual to another. For the purpose of the present
invention, the melanoma cell line used as a source of
melanoma-specific CTL epitopes may be any melanoma cell line;

similarly, the Class I MHC (HLA) molecule may be any such
molecule borne by a melanoma which is capable of binding to
and presenting a melanoma-specific epitope, including, but
not limited to, the various allelic forms of Class I MHC
5 molecules, including but not limited to those enumerated in
Table I. Among the Class I molecules, the principal genetic
loci are denoted as HLA-A, HLA-B, and HLA-C. The preferred
epitopic sequence may vary depending on the restriction
system.

10 Application of active specific immunotherapy to a
heterogeneous melanoma patient population would be
facilitated by identification of CTL epitopes presented in
association with a wide range of class I MHC molecules.
Besides HLA-A2, the most commonly expressed class I MHC
15 molecules are A1 and A3, then B7 and B8. Approximately 90% of
the melanoma patient population should express one or more of
these molecules or HLA-A2. Peptides from MAGE-1 and MAGE-3
have been identified as HLA-A1-restricted CTL epitopes, and a
few peptides have been identified for some of the less common
20 MHC molecules, including A24, A31, and B44. Little work has
been done toward identification of HLA-A3-restricted
responses, and-except for the peptides from MAGE proteins -
little work has been done toward identification of HLA-A1-
restricted responses.

25 Preferably, the epitope is one restricted by one of the
more prevalent forms (in the melanoma patient population) of
these loci. The loci HLA-A1, HLA-A2, HLA-A3, HLA-B7 and HLA-
B8 are of greatest interest. Within HLA-A2, HLA-A2.1 is of
particular interest. However, over half of patients do not
30 express HLA-A2.1, and it is therefore desirable to identify
immunodominant CTL peptide epitopes restricted by common MHC
molecules other than HLA-A2.1, e.g., HLA-A1.

Preferably, the CTL epitopes of the present invention,
in the cytotoxicity assay described hereafter, when used in
35 oligopeptide form to reconstitute epitopes for suitable CTL,
achieve, at the dosage resulting in maximal lysis of target
cells exposed to the stimulated CTL, a percentage lysis of
target cells which is at least 10 percentage points higher

(more preferably, at least 20 points higher) the background level of lysis of the target cells by the CTLs (i.e., in absence of the peptide).

Generally speaking, in addition to epitopes which are
5 identical to the naturally occurring disease-specific epitopes, the present invention embraces epitopes which are substantially identical with such epitopes, and therefore disease-specific in their own right.

The term "substantially identical", when used in
10 connection with amino acid sequences, refers to sequences which are substantially identical to or similar in sequence with each other, giving rise to a homology in conformation and thus to similar (or improved) biological activity. The term is not intended to imply a common evolution of the
15 sequences.

An epitope is considered substantially identical to a reference epitope if it has at least 10% of an immunological activity of the reference epitope and differs from the reference epitope by no more than one non-conservative
20 substitution not suggested by a known binding motif of the pertinent MHC molecule. Any number of highly conservative, conservative or semi-conservative substitutions, or non-conservative substitutions suggested by known binding motifs, subject to the activity limitation, are permitted.

25 Kast, et al., J. Immunol, 152:3904-12 (1994) sets forth HLA-A specific peptide binding motifs for the HLA molecules A1, A2.1, A3, A11 and A24. Engelhard, et al., in Sette, ed., Naturally Processed Peptides, 57:39-62 (1993) explored the features that determined binding to HLA-A2.1 and HLA-B7. See
30 also Hobohim et al; Eur. J. Immunol., 23:1271-6 (1993); Kawakami, et al., J. Immunol., 154:3961-8 (1995). Based on these and other sources, the preferred and tolerated AAs for various HLA molecules include (but are not limited to) the following:

35 Table 10

Molecule	Position	Preferred AA	tolerated AA
A1	2	T, S, M	

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		3	D, E	A, S
		9	Y	
	A2.1	2	L, M	I, V, A, T
		9	L, V, I	A, M, T
5	A3	2	L, M, I, V, S A, T, F	C, G, D
		9	K, R, Y, H, F	A
	A11	2	M, L, I, V, S A, T, G, N	C, D, F
10		9	K	R, H, Y
	A24	2	Y, F, W	M
		9	F, L, I, W	
	B7	1	A	M, S, R, L
		2	P	V
15		3	R	A, K, S, M
		9	L	I, A, V
	B8	3	K	not known
		5	K	not known
		9	L	not known
20	B27	2	R	not known
		9	R, K, H	not known
	B35	2	P	not known
		9	Y	not known
	B53	2	P	not known

25   If a position is not listed, studies revealed a greater variability of AAs than for the listed positions. For listed positions, AAs not listed may be tolerated, especially if they are conservative or semi-conservative substitutions for "preferred" or "tolerated" AAs.

30   Substantially identical peptide epitopes may be identified by a variety of techniques, some of which do not depend on preexisting knowledge of the binding motif. Thus, it is known in the art that one may synthesize all possible single substitution mutants of a known peptide epitope. For

35 a nonpeptide, there are (20x9-1=179) such mutants. Geysen, et al., Proc Nat. Acad. Sci. (USA), 81:3998-4002 (1984). While the effects of different substitutions are not always additive, it is reasonable to expect that two favorable or neutral single substitutions at different residue positions

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in the epitope can safely be combined in most cases.

One may also synthesize a family of related single or multiple substitution mutants, present the mixture to the HLA-A2.1 positive lymphoblastoid cell line T2 (or other cell line capable of presenting melanoma-specific CTL epitopes), and expose the T2 cells to melanoma-specific CTLs. If the T2 cells are lysed, the effective epitopes may be identified either by direct recovery from the T2 cells or by a progressive process of testing subsets of the effective peptide mixtures. Methods for the preparation of degenerate peptides are described in Rutter, USP 5,010,175, Haughten, et al., Proc. Nat. Acad. Sci. (USA), 82:5131-35 (1985), Geysen, et al., Proc. Nat. Acad. Sci. (USA), 81:3998-4002 (1984); WO86/06487; WO86/00991.

Multiple mutagenesis may be used to screen a few residue positions intensely or a larger number of positions more diffusely. One approach is to explore at least a representative member of each a.a. type at each position, e.g., one representative of each of exchange groups I-V as hereafter defined. Preferably, Gly and Pro are screened in addition to one other group I residue. Preferably, at least one screened residue is an H-bonding resiude. If a positive mutant features a particular representative, like amino acids can be explored in a subsequent library. If, for example, a Phe substitution improves binding, Tyr and Trp can be examined in the next round.

For one preferred A1 peptide a possible multiple mutagenesis strategy would be

Lys	Cys	Asp	Ile	Cys	Thr	Asp	Glu	Tyr
Arg	Thr	Glu	Leu	Thr	Ala	Glu	Asp	Phe
His	Ser		Val	Ser	Ser			Trp
	Ala		Met	Ala	Pro			
	Met			Gly	Gly			
	Gly			Met				

These strategies take into account conservative substitutions for the wild type AAs, and the known A1, A2.1 and A3 binding motifs, and preferred method of cysteine depletion.

The person of ordinary skill in the art, in determining which residues to vary, may also make comparisons of the

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sequences of the naturally processed MHC associated peptides, and may obtain 3D structures of the MHC: peptide: TCR complexes, in order to identify residues involved in MHC or TCR binding. Such residues may either be left alone, or
5   judiciously mutated in an attempt to enhance MHC or TCR binding.

It is also possible to predict substantially homologous epitopes by taking into account studies of sequence variations in families of naturally occurring homologous
10 proteins. Certain amino acid substitutions are more often tolerated than others, and these are often correlatable with similarities in size, charge, etc. between the original amino acid and its replacement. Insertions or deletions of amino acids may also be made. N- and C-terminal truncations or
15 extensions are more likely to be tolerated than internal deletions or insertions. With regard to truncation, the peptide may be truncated by one or more amino acids and still be substantially homologous, however, it cannot be fewer than five amino acids. Extensions are permissible, however, note
20 that larger peptides are digested _in vivo_ prior to presentation.

Conservative substitutions may be made in the amino acid sequence of the proteins of interest without compromising the desired properties of the peptides, i.e., induction of
25 cytotoxic T-lymphocytes in a patient when administered thereto.

Conservative substitutions are herein defined as exchanges within one of the following five groups:

I.   Small aliphatic, nonpolar or slightly
30   polar residues:
         Ala, Ser, Thr, Pro, Gly

II. Polar, negatively charged residues: and
         their amides
         Asp, Asn, Glu, Gln

35   III. Polar, positively charged residues:
         His, Arg, Lys

IV. Large, aliphatic, nonpolar residues:
         Met, Leu, Ile, Val, Cys

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V.   Large, aromatic residues:

        Phe, Tyr, Trp

        Within the foregoing groups, the following substitutions
are considered "highly conservative":

5   Asp/Glu

                His/Arg/Lys

                Phe/Tyr/Trp

                Met/Leu/Ile/Val

        Semi-conservative substitutions are defined to be
10   exchanges between two of groups (I)-(V) above which are
limited to supergroup (A), comprising (I), (II) and (III)
above, or to supergroup (B), comprising (IV) and (V) above.
Also, Ala is considered a semi-conservative substitution for
all non group I amino acids.

15   It will be appreciated that highly conservative
substitutions are less likely to affect activity than other
conservative substitutions, conservative substitutions are
less likely to affect activity than merely semi-conservative
substitutions, and semi-conservative substitutions less so
20   than non-conservative substitutions.

        Although a substitution mutant, either single or
multiple, of the peptides of interest may not have quite the
potency of the original peptide, such a mutant may well be
useful.

25   Substitutions are not limited to the genetically
encoded, or even the naturally occurring amino acids. When
the epitope is prepared by peptide synthesis, the desired
amino acid may be used directly. Alternatively, a genetical-
ly encoded amino acid may be modified by reacting it with an
30   organic derivatizing agent that is capable of reacting with
selected side chains or terminal residues. The following
examples of chemical derivatives are provided by way of
illustration and not by way of limitation.

        Aromatic amino acids may be replaced with D- or
35   L-naphylalanine, D- or L-Phenylglycine, D- or L-2-thieney-
lalanine, D- or L-1-, 2-, 3- or 4-pyreneylalanine, D- or
L-3-thieneylalanine, D- or L-(2-pyridinyl)-alanine, D- or
L-(3-pyridinyl)-alanine, D- or L-(2-pyrazinyl)-alanine, D- or

L-(4-isopropyl)-phenylglycine, D-(trifluoromethyl)- phenyl-
glycine, D-(trifluoromethyl)-phenylalanine, D-p-fluoro-
phenylalanine, D- or L-p-biphenylphenylalanine, D- or
L-p-methoxybiphenylphenylalanine, D- or L-2-indole-
5 (alkyl)alanines, and D- or L-alkylainines where alkyl may be
substituted or unsubstituted methyl, ethyl, propyl, hexyl,
butyl, pentyl, iso-propyl, iso-butyl, sec-isotyl, iso-pentyl,
non-acidic amino acids, of C1-C20.

Acidic amino acids can be substituted with non-
10 carboxylate amino acids while maintaining a negative charge,
and derivatives or analogs thereof, such as the non-limiting
examples of (phosphono)-alanine, glycine, leucine,
isoleucine, threonine, or serine; or sulfated (e.g., $-SO_3H$)
threonine, serine, tyrosine.

15   Other substitutions may include unnatural hyroxylated
amino acids made by combining "alkyl" (as defined and
exemplified herein) with any natural amino acid. Basic amino
acids may be substituted with alkyl groups at any position of
the naturally occurring amino acids lysine, arginine,
20 ornithine, citrulline, or (guanidino)-acetic acid, or other
(guanidino)alkyl-acetic acids, where "alkyl" is define as
above. Nitrile derivatives (e.g., containing the CN-moiety
in place of COOH) may also be substituted for asparagine or
glutamine, and methionine sulfoxide may be substituted for
25 methionine. Methods of preparation of such peptide
derivatives are well known to one skilled in the art.

In addition, any amide linkage can be replaced by a
ketomethylene moiety, e.g. $(-C(=O)-CH_2-)$ for $(-(C=O)-NH-)$.
Such derivatives are expected to have the property of
30 increased stability to degradation by enzymes, and therefore
possess advantages for the formulation of compounds which may
have increased _in vivo_ half lives, as administered by oral,
intravenous, intramuscular, intraperitoneal, topical, rectal,
intraocular, or other routes.

35   In addition, any amino acid can be replaced by the same
amino acid but of the opposite chirality. Thus, any amino
acid naturally occurring in the L-configuration (which may
also be referred to as the R or S configuration, depending

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upon the structure of the chemical entity) may be replaced
with an amino acid of the same chemical structural type, but
of the opposite chirality, generally referred to as the D-
amino acid but which can additionally be referred to as the
5   R- or the S-, depending upon its composition and chemical
configuration. Such derivatives have the property of greatly
increased stability to degradation by enzymes, and therefore
are advantageous in the formulation of compounds which may
have longer _in vivo_ half lives, when administered by oral,
10 intravenous, intramuscular, intraperitoneal, topical, rectal,
intraocular, or other routes.

The thiol group of cysteine reacts very rapidly with
alkyl halides, such as iodoacetate, iodoacetamide, methyl
iodine, and so on, to give the corresponding stable alkyl
15 (substituted or unsubstituted) derivatives, such as $-CH_2-S-$
CH_3. The thiol group can also add across double bonds such as
those of N-ethylmaleimide or of maleic anhydride, and it can
open the ring of ethyleneimine, providing a new site for
tryptic cleavage. Thiols form complexes with various metal
20 (especially mercury, silver, arsenic, copper, iron, zinc,
cobalt, molybdenum, manganese and cadmium ions) and
organometal ions (e.g., $R-Hg^+$, such as para-mercuribenzoic
acid).

The thiol group may be oxidized to yield a disulfide
25 bond or a sulfonate. A thiol may be converted to a disulfide
by thiol-disulfide exchange, for example, exchange with an
aromatic disulfide such as dithionitrobenzoic acid (DTNB) or
Ellman's reagnet. Of course, a cysteine residue may be
disulfide bonded to a cysteine residue in the same or a
30 different peptide, or to a free cysteine. By way of further
examples, some of which are already embraced by the general
discussion above, cysteinyl residues may be reacted with
alpha-haloacetates (and corresponding amines), such as
2-chloroacetic acid or chloroacetamide, to give carboxymethyl
35 or carboxyamidomethyl derivatives. Cysteinyl residues may
also be derivatized by reaction with compounds such as
bromotrifluoroacetone, alpha-bromo-beta-(5-imidozoyl)-
propionic acid, chloroacetyl phosphate, N-alkylmaleimides,

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3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide,
p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or
chloro-7-nitrobenzo-2-oxa-1,3-diazole.

5   Histidyl residues may be derivatized by reaction with
compounds such as diethylprocarbonate e.g., at pH 5.5-7.0
because this agent is relatively specific for the histidyl
side chain, and para-bromophenacyl bromide may also be used;
e.g., where the reaction is preferably performed in 0.1 M
sodium cacodylate at pH 6.0.

10   Lysinyl and amino terminal residues may be reacted with
compounds such as succinic or other carboxylic acid
anhydrides. Derivatization with these agents is expected to
have the effect of reversing the charge of the lysinyl
residues. Other suitable reagents for derivatizing alp-

15 ha-amino-containing residues include compounds such as
imidoesters/e.g., as methyl picolinimidate; pyridoxal
phosphate; pyridoxal; chloroborohydride;
trinitrobenzenesulfonic acid; O-methylisourea; 2,4
pentanedione; and transaminase-catalyzed reaction with

20 glyoxylate.

       Arginyl residues may be modified by reaction with one or
several conventional reagents, among them phenylglyoxal,
2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin
according to known method steps. Derivatization of arginine

25 residues requires that the reaction be performed in alkaline
conditions because of the high pKa of the guanidine
functional group. Furthermore, these reagents may react with
the groups of lysine as well as the arginine epsilon-amino
group.

30   The specific modification of tyrosyl residues per se is
well-known, such as for introducing spectral labels into
tyrosyl residues by reaction with aromatic diazonium
compounds or tetranitromethane. N-acetylimidizol and
tetranitromethane may be used to form O-acetyl tyrosyl

35 species and 3-nitro derivatives, respectively.

       Carboxyl side groups (aspartyl or glutamyl) may be
selectively modified by reaction with carbodiimides
(R'-N-C-N-R') such as 1-cyclohexyl-3-(2-morpholinyl-

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(4-ethyl) carbodiimide or 1- ethyl-3-(4-azonia-4,4-
dimethylpentyl) carbodiimide. Furthermore, aspartyl and
glutamyl residues may be converted to asparaginyl and
glutaminyl residues by reaction with ammonium ions.

5   Glutaminyl and asparaginyl residues may be readily
deamidated to the corresponding glutamyl and aspartyl
residues. Alternatively, these residues may be deamidated
under mildly acidic conditions. Either form of these
residues falls within the scope of the present invention.

10   Derivatization with bifunctional agents is useful for
cross-linking the peptide to a water-insoluble support matrix
or to other macromolecular carriers, according to known
method steps. Commonly used cross-linking agents include,
e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde,
15 N-hydroxysuccinimide esters, for example, esters with
4-azidosalicylic acid, homobifunctional imidoesters, in-
cluding disuccinimidyl esters such as 3,3'- dithiobis(suc-
cinimidylpropionate), and bifunctional maleimides such as
bis-N-maleimido-1,8-octane. Derivatizing agents such as
20 methyl-3-[(p-azidophenyl)dithio]propioimidate yield
photoactivatable intermediates that are capable of forming
crosslinks in the presence of light. Alternatively, reactive
water-insoluble matrices such as cyanogen bromide-activated
carbohydrates and the reactive substrates described in U.S.
25 Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642;
4,229,537; and 4,330,440 (which are herein incorporated
entirely by reference), may be employed for protein
immobilization.

        Other modifications include hydroxylation of proline and
30 lysine, phosphorylation of hydroxyl groups of seryl or
threonyl residues, methylation of the alpha-amino groups of
lysine, arginine, and histidine side chains (Creighton, T.E.,
Proteins: Structure and Molecule Properties, W.H. Freeman &
Co., San Francisco, pp. 79-86 (1983)), acetylation of the
35 N-terminal amine, methylation of main chain amide residues
(or substitution with N-methyl amino acids) and, in some
instances, amidation of the C-terminal carboxyl groups,
according to known method steps. Glycosylation is also

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possible.

Derivatized moieties may impart altered affinity for their target, altered immunogenicity, or improved solubility, absorption, biological half life, and the like, or attenuated

5   undesirable side effects. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980).

Modifications are not limited to the side chains of the

10 amino acids. One may also modify the peptidyl linkage itself, e.g., -NRCO- (where R is alkyl or aryl), instead of -NHCO-, as in the so-called "peptoids."

The peptides may also comprise isoteres of two or more residues in the immunogenic peptide. An isotere as defined

15 here is a sequence of two or more residues that can be sustituted for a second sequence because the steric conformation of the first sequence fits a binding site specific for the second sequence. The term specifically includes peptide backbone modifications well known to those

20 skilled in the art. Such modifications include modifications of the amide nitrogen, the α-carbon, amide carbonyl, complete replacement of the amide bond, extensions, deletions or backbone crosslinks. See, generally, Spatola, Chemistry and Biochemistry of Amino Acids, peptides and Proteins, Vol. VII

25 (Weinstein ed., 1983).

It is also possible to construct and use so-called peptide mimetics whose conformation is similar to that of a peptide but do not have a peptide-like molecular formula. In effect, in a mimetic, all of the residues of the peptide are

30 replaced by one or more isoteres as defined above.

The Disease-Specific Immunogen

The disease-specific immunogen of the present invention is a molecule corresponding to or otherwise comprising a disease-specific CTL epitope as previously described. The

35 immunogen may comprise one or more disease-specific CTL epitopes, which may be the same or different. Preferably, the immunogen is chosen so that at least one epitope is

effective in each of two or more restriction systems, e.g.,
HLA-A2 and HLA-A3. In some instances, a single epitope may
be effective in more than one restriction system. For
example HLA-A2 and HLA-69, or HLA-A3 and HLA-A11, are pairs

5   of MHC molecules having similar peptide binding motifs.
Otherwise, for the immunogen to be effective in more than one
restriction system, two or more epitopes (at least one for
each MHC molecule of interest) will need to be provided.
These epitopes may be separate or overlapping.

10   It should be noted that instead of linking epitopes
within a single immunogen, the compositions of the present
invention may include two or more immunogens which present
different epitopes.

        If the immunogen comprises a plurality of such epitopes,
15 they may be linked directly, or through a spacer of some
kind, or by noncovalent means such as an avidin:biotin
complex. The immunogen may take any form that is capable of
eliciting a suitable cytotoxic immune response. By way of
example and not of limitation, the immunogen may be a fusion

20 of a plurality of CTL epitopes which is sufficiently large to
be immunogenic, a conjugate of one or more epitopes to a
soluble immunogenic macromolecular carrier, such as serum
albumin, keyhole limpet hemocyanin, or dextran, a recombinant
virus engineered to display the epitope on its surface, or a

25 conjugate of a plurality of epitopes to a branched lysine
core structure, a so-called "multiple antigenic peptide" (see
Posnett, et al., J. Biol. Chem., 263:1719-25, 1988).

        The immunogenic conjugate may also comprise moieties
intended to enhance the immune response, such as a T helper

30 peptide, a cytokine or an adjuvant; a targeting agent, such
as an antibody or receptor ligand or ligand analogue; or a
stabilizing agent, such as a lipid.

        For instance, the ability of the peptides to induce CTL
activity can be enhanced by linkage to a sequence which

35 contains at least one epitope that is capable of inducing a T
helper cell response. Particularly preferred immunogenic
peptides/T helper conjugates are linked by a spacer molecule.
The spacer is typically comprised of relatively small,

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neutral molecules, such as amino acids or amino acid
mimetics, which are substantially uncharged under
physiological conditions.   The spacers are typically selected
from, e.g., Ala, Gly, or other neutral spacers of nonpolar
5   amino acids or neutral polar amino acids.   It will be
understood that the optionally present spacer need not be
comprised of the same residues and thus may be a hetero- or
homo-oligomer.   When present, the spacer will usually be at
least one or two residues, more usually three to six
10   residues.   Alternatively, the CTL peptide may be linked to
the T helper peptide without a spacer.

The immunogenic peptide may be linked to the T helper
peptide either directly or via a spacer either at the amino
or carboxy terminus of the CTL peptide.   The amino terminus
15   of either the immunogenic peptide or the T helper peptide may
be acylated.

Melanoma-specific CTL epitopes are of particular
interest.   Besides one or more of the novel melanoma-specific
CTL epitopes described herein, the immunogen may present one
20   or more such epitopes already known in the art, such as the
following:

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Table A.   Peptide epitopes for human tumor-specific CTL

Protein	MHC restriction	Peptide sequence	Tumor type
Tyrosinase	A2	MLLAYLYCL	Melanoma
Tyrosinase	A24	AFLPWHRLF, AFLPWHRLFL	Melanoma
Tyrosinase	B44	SEIWRDIDF	Melanoma
gp100/Pmel17	A2	KTWGQYWQV	Melanoma
gp100/Pmel17	A2	ITDQVPFSV	Melanoma
gp100/Pmel17	A2	VLYRYGSFSV	Melanoma
gp100/Pmel17	A2	LLDGTATLRL	Melanoma
MART-1/MelanA	A2	AAGIGILTV	Melanoma
MART-1/MelanA	A2	ILTVILGVL	Melanoma
gp75/TRP-1	A31	- - - -	Melanoma
MAGE-1	A1	EADPTGHSY	Melanoma, other tumors [1]
MAGE-1	Cw*1601	SAYGEPRKL	Melanoma, other tumors [1]

MAGE-3	A1	EVDPIGHLY	Melanoma, other tumors [2]
MAGE-3	A2	FLWGPRALV	Melanoma, other tumors [2]
BAGE	Cw*1601	AARAVFLAL	Melanoma, other tumors [3]
GAGE-1,2	Cw6	YRPRPRRY	Melanoma, other tumors 4
HER-2/neu	A2	KIFGSLAFL, VMAGVGSPYV	Ovarian Cancer
HER-2/neu	A2	IISAVVGIL	Ovarian Cancer, NSCLC
CEA	A2	YLSGANLNL	Colon Cancer
p15	A24	(E)AYGLDFYIL	Melanoma and normal tissues
43kD protein	A2	QDLTMKYQIF	Melanoma

MUM-1 gene product mutated across intron/exon junction	B*4402	EEKLIVVLF [5]	Melanoma
mutated beta-catenin	A24	SYLDSGIHF [6]	Melanoma

[1]   MAGE-1: expressed in Melanoma (36%), Bladder CA (19%), Breast CA (18%), Head & neck CA (25%), Non-small cell lung CA (NSCLC, 34%), Sarcomas (11%), Prostate CA (15%) [50]

[2]   MAGE-3: expressed in Melanoma (65%), Bladder CA (34%), Breast CA (11%), Head & neck CA (48%), Non-small cell lung CA (NSCLC, 31%), Sarcomas (11%), Prostate CA (15%) [50]

[3]   BAGE: expressed in Melanoma (22%), Bladder CA (15%), Breast CA (10%), Head and neck CA (<10%), NSCLC (<10%) [50]

[4]   GAGE-1, -2: expressed in Melanoma (24%), Bladder CA (12%), Breast CA (9%), Head & neck CA (19%), NSCLC (19%), Sarcomas (25%), Prostate cancers (10%) [50]

[5]   Isoleucine (I) at position 5 is the result of mutation. The wild type sequence is EEKLSVVLF.

[6]   Phenylalanine (F) at pos. 9 is the result of mutation. The wild type sequence is SYLDSGIHS.

Note that the first entry on this table is a tyrosinase A2 epitope which contains a cysteine, and is therefore a candidate for cysteine depletion.

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If it is desirable to present more than one CTL epitope,
rather than presenting all of the epitopes on a single
immunogen, they may be presented on two or more different
immunogens. These may be administered separately, or as part
5   of a mixture, e.g., a mixture of epitopic peptides.

Mode of Production

The peptide portion of the immunogens of the present
invention may be produced by any conventional technique,
including

10   (a)   nonbiological synthesis by sequential
                  coupling of component amino acids,
            (b)   production by recombinant DNA techniques
                  in a suitable host cell, and
            (c)   chemical or enzymatic modification of a
15   sequence made by (a) or (b) above.

Gene Expression. The peptides disclosed herein may be
produced, recombinantly, in a suitable host, such as bacteria
from the genera Bacillus, Escherichia, Salmonella, Erwinia,
and yeasts from the genera Hansenula, Kluyveromyces, Pichia,
20 Rhinosporidium, Saccharomyces, and Schizosaccharomyces, or
cultured mammalian cells such as COS-1. The more preferred
hosts are microorganisms of the species *Pichia pastoris,*
Bacillus subtilis, Bacillus brevis, Saccharomyces cerevisiae,
Escherichia coli and *Yarrowia lipolytica.* Any promoter,
25 regulatable or constitutive, which is functional in the host
may be used to control gene expression.

These sequences may be constructed in such a manner,
including the appropriate expression systems for use in gene
therapy procedures. Because several different nucleotide
30 sequences may encode a single amino acid, alternate DNA
sequences may also encode these peptides.

Standard reference works setting forth the general
principles of recombinant DNA technology include Watson,
J.D., et al., Molecular Biology of the Gene, Volumes I and
35 II, The Benjamin/Cummings Publishing Company, Inc.,
publisher, Menlo Park, CA (1987); Darnell, J.E., et al.,
Molecular Cell Biology, Scientific American Books, Inc.,

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publisher, New York, N.Y. (1986); Lewin, B.M., Genes II, John Wiley & Sons, publishers, New York, N.Y. (1985); Old, R.W., et al., Principles of Gene Manipulation: An Introduction to Genetic Engineering, 2d edition, University

5   of California Press, publisher, Berkeley, CA (1981); Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989); and Ausubel, et al., Current Protocols in Molecular Biology, Wiley Interscience, N.Y., (1987, 1992). These

10 references are herein entirely incorporated by reference.
        Chemical Peptide Synthesis. Chemical peptide synthesis is a rapidly evolving area in the art, and methods of solid phase peptide synthesis are well-described in the following references, hereby entirely incorporated by reference:

15 (Merrifield, B., J. Amer. Chem. Soc. 85:2149-2154 (1963); Merrifield, B., Science 232:341-347 (1986); Wade, J.D., et al., Biopolymers 25:S21-S37 (1986); Fields, G.B., Int. J. Polypeptide Prot. Res. 35:161 (1990); MilliGen Report Nos. 2 and 2a, Millipore Corporation, Bedford, MA, 1987) Ausubel, et

20 al, supra, and Sambrook, et al, supra.
        In general, as is known in the art, such methods involve blocking or protecting reactive functional groups, such as free amino, carboxyl and thio groups. After polypeptide bond formation, the protective groups are removed (or de-protect-

25 ed). Thus, the addition of each amino acid residue requires several reaction steps for protecting and deprotecting. Current methods utilize solid phase synthesis, wherein the C-terminal amino acid is covalently linked to an insoluble resin particle large enough to be separated from the fluid

30 phase by filtration. Thus, reactants are removed by washing the resin particles with appropriate solvents using an automated programmed machine. The completed polypeptide chain is cleaved from the resin by a reaction which does not affect polypeptide bonds.

35   In the more classical method, known as the "tBoc method," the amino group of the amino acid being added to the resin-bound C-terminal amino acid is blocked with tert-butyloxycarbonyl chloride (tBoc). This protected amino

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acid is reacted with the bound amino acid in the presence of
the condensing agent dicyclohexylcarbodiimide, allowing its
carboxyl group to form a polypeptide bond the free amino
group of the bound amino acid. The amino-blocking group is
5   then removed by acidification with trifluoroacetic acid
(TFA); it subsequently decomposes into gaseous carbon dioxide
and isobutylene. These steps are repeated cyclically for
each additional amino acid residue. A more vigorous
treatment with hydrogen fluoride (HF) or trifluoro-
10   methanesulfonyl derivatives is common at the end of the
synthesis to cleave the benzyl-derived side chain protecting
groups and the polypeptide-resin bond.

     More recently, the preferred "Fmoc" technique has been
introduced as an alternative synthetic approach, offering
15   milder reaction conditions, simpler activation procedures and
compatibility with continuous flow techniques. This method
was used, e.g., to prepare the peptide sequences disclosed in
the present application. Here, the α-amino group is
protected by the base labile 9-fluorenylmethoxycarbonyl
20   (Fmoc) group. The benzyl side chain protecting groups are
replaced by the more acid labile t-butyl derivatives.
Repetitive acid treatments are replaced by deprotection with
mild base solutions, e.g., 20% piperidine in dimethyl-
formamide (DMF), and the final HF cleavage treatment is
25   eliminated. A TFA solution is used instead to cleave side
chain protecting groups and the peptide resin linkage
simultaneously.

     At least three different peptide-resin linkage agents
can be used: substituted benzyl alcohol derivatives that can
30   be cleaved with 95% TFA to produce a peptide acid, methanolic
ammonia to produce a peptide amide, or 1% TFA to produce a
protected peptide which can then be used in fragment
condensation procedures, as described by Atherton, E., et
al., J. Chem. Soc. Perkin Trans. 1:538-546 (1981) and
35   Sheppard, R.C., et al., Int. J. Polypeptide Prot. Res.
20:451-454 (1982). Furthermore, highly reactive Fmoc amino
acids are available as pentafluorophenyl esters or dihydro-
oxobenzotriazine esters derivatives, saving the step of

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activation used in the tBoc method.

Pharmaceutical Methods and Preparations

        The preferred animal subject of the present invention is
a primate mammal. By the term "mammal" is meant an
5   individual belonging to the class Mammalia, which, of course,
includes humans. The invention is particularly useful in the
treatment of human subjects, although it is intended for
veterinary uses as well. By the term "non-human primate" is
intended any member of the suborder Anthropoidea except for
10 the family Hominidae. Such non-human primates include the
superfamily Ceboidea, family Cebidae (the New World monkeys
including the capuchins, howlers, spider monkeys and squirrel
monkeys) and family Callithricidae (including the marmosets);
the superfamily Cercopithecoidea, family Cercopithecidae
15 (including the macaques, mandrills, baboons, proboscis
monkeys, mona monkeys, and the sacred hunaman monkeys of
India); and superfamily Hominoidae, family Pongidae
(including gibbons, orangutans, gorillas, and chimpanzees).
The rhesus monkey is one member of the macaques.
20   The term "protection", as used herein, is intended to
include "prevention," "suppression" and "treatment."
"Prevention" involves administration of the protein <u>prior to
the induction</u> of the disease. "Suppression" involves
administration of the composition <u>prior to the clinical
25 appearance</u> of the disease. "Treatment" involves
administration of the protective composition <u>after the
appearance</u> of the disease.
        It will be understood that in human and veterinary
medicine, it is not always possible to distinguish between
30 "preventing" and "suppressing" since the ultimate inductive
event or events may be unknown, latent, or the patient is not
ascertained until well after the occurrence of the event or
events. Therefore, it is common to use the term
"prophylaxis" as distinct from "treatment" to encompass both
35 "preventing" and "suppressing" as defined herein. The term
"protection," as used herein, is meant to include
"prophylaxis." It should also be understood that to be

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useful, the protection provided need not be absolute,
provided that it is sufficient to carry clinical value. An
agent which provides protection to a lesser degree than do
competitive agents may still be of value if the other agents

5 are ineffective for a particular individual, if it can be
used in combination with other agents to enhance the level of
protection, or if it is safer than competitive agents.

The composition may be administered parentally or
orally, and, if parentally, either systemically or topically.

10 Parenteral routes include subcutaneous, intravenous
intradermal, intramuscular, intraperitoneal, intranasal,
transdermal, or buccal routes. One or more such routes may
be employed. Parenteral administration can be, e.g., by
bolus injection or by gradual perfusion over time.

15 Alternatively, or concurrently, administration may be by the
oral route. The immunization is preferably accomplished
initially by intramuscular injection followed by intradermal
injection, although any combination of intradermal and
intramuscular injections may be used.

20   It is understood that the suitable dosage of a immunogen
of the present invention will be dependent upon the age, sex,
health, and weight of the recipient, kind of concurrent
treatment, if any, frequency of treatment, and the nature of
the effect desired. However, the most preferred dosage can

25 be tailored to the individual subject, as is understood and
determinable by one of skill in the art, without undue
experimentation. This will typically involve adjustment of a
standard dose, e.g., reduction of the dose if the patient has
a low body weight.

30   Prior to use in humans, a drug will first be evaluated
for safety and efficacy in laboratory animals. In human
clinical studies, one would begin with a dose expected to be
safe in humans, based on the preclinical data for the drug in
question, and on customary doses for analogous drugs (if

35 any). If this dose is effective, the dosage may be
decreased, to determine the minimum effective dose, if
desired. If this dose is ineffective, it will be cautiously
increased, with the patients monitored for signs of side

effects. See, e.g., Berkow, et al., eds., The Merck Manual, 15th edition, Merck and Co., Rahway, N.J., 1987; Goodman, et al., eds., Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th edition, Pergamon Press, Inc., Elmsford,

5 N.Y., (1990); Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD. (1987), Ebadi, Pharmacology, Little, Brown and Co., Boston, (1985), which references and references cited therein, are entirely

10 incorporated herein by reference.

The total dose required for each treatment may be administered in multiple doses (which may be the same or different) or in a single dose, according to an immunization schedule, which may be predetermined or ad hoc. The schedule

15 is selected so as to be immunologically effective, i.e., so as to be sufficient to elicit an effective CTL response to the antigen and thereby, possibly in conjunction with other agents, to provide protection. The doses adequate to accomplish this are defined as "therapeutically effective

20 doses." (Note that a schedule may be immunologically effective even though an individual dose, if administered by itself, would not be effective, and the meaning of "therapeutically effective dose" is best interpreted in the context of the immunization schedule.) Amounts effective for

25 this use will depend on, e.g., the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician, but generally range for the initial immunization

30 (that is for therapeutic or prophylactic administration) from about 1.0 μg to about 5000 μg of peptide for a 70 kg patient, followed by boosting dosages of from about 1.0 μg to about 1000 μg of peptide pursuant to a boosting regimen over weeks to months depending upon the patient's response and condition

35 by measuring specific CTL activity in the patient's blood. It must be kept in mind that the peptides and compositions of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life

threatening situations. In such cases, in view of the
minimization of extraneous substances and the relative
nontoxic nature of the peptides, it is possible and may be
felt desirable by the treating physician to administer
5   substantial excesses of these peptide compositions.

The doses may be given at any intervals which are
effective. If the interval is too short, immunoparalysis or
other adverse effects can occur. If the interval is too
long, immunity may suffer. The optimum interval may be
10   longer if the individual doses are larger. Typical intervals
are 1 week, 2 weeks, 4 weeks (or one month), 6 weeks, 8 weeks
(or two months) and one year. The appropriateness of
administering additional doses, and of increasing or
decreasing the interval, may be reevaluated on a continuing
15   basis, in view of the patient's immunocompetence (e.g., the
level of antibodies to melanoma-associated antigens).

The concentration of CTL stimulatory peptides of the
invention in the pharmaceutical formulations can vary widely,
i.e., from less than about 0.1%, usually at or at least about
20   2% to as much as 20% to 50% or more by weight, and will be
selected primarily by fluid volumes, viscosities, etc., in
accordance with the particular mode of administration
selected.

In one embodiment, the immunogen is dissolved or
25   suspended in an aqueous carrier. A variety of aqueous
carriers may be used, e.g., water, buffered water, 0.9%
saline, 0.3% glycine, hyaluronic acid and the like. These
compositions may be sterilized by conventional, well known
sterilization techniques, or may be sterile filtered. The
30   resulting aqueous solutions may be packaged for use as is, or
lyophilized, the lyophilized preparation being combined with
a sterile solution prior to administration. The compositions
may contain pharmaceutically acceptable auxiliary substances
as required to approximate physiological conditions, such as
35   pH adjusting and buffering agents, tonicity adjusting agents,
wetting agents and the like, for example, sodium acetate,
sodium lactate, sodium chloride, potassium chloride, calcium
chloride, sorbitan monolaurate, triethanolamine oleate, etc.

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The peptides of the invention may also be administered via liposomes, which serve to target the peptides to a particular tissue, as well as increase the half-life of the peptide composition. Liposomes include emulsions, foams,
5 micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the peptide to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to, e.g., a receptor prevalent among melanocytes
10 or melanomas, or with other therapeutic or immunogenic compositions. Thus, liposomes filled with a desired peptide of the invention can be directed to the site of target cells, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions. Liposomes for
15 use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the
20 liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., Ann. Rev. Biophys. Bioeng. 9:467 (1980), U.S. Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019369, incorporated herein by reference.
25   For targeting to the melanoma cells, a ligand to be incorporated into the liposome can include, e.g., antibodies or fragments thereof specific for cell surface determinants of the desired melanoma cells. A liposome suspension containing a peptide may be administered intravenously,
30 locally, topically, etc. in a dose which varies according to, inter alia, the manner of administration, the peptide being delivered, and the stage of the disease being treated.
      For solid compositions, conventional nontoxic solid carriers may be used which include, for example,
35 pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic

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composition is formed by incorporating any of the normally
employed excipients, such as those carriers previously
listed, and generally 10-95% of active ingredient, that is,
one or more peptides of the invention, and more preferably at
5   a concentration of 25%-75%.

For aerosol administration, the immunogenic peptides are
preferably supplied in finely divided form along with a
surfactant and propellant. Typical percentages of peptides
are 0.01%-20% by weight, preferably 1%-10%. The surfactant
10 must, of course, be nontoxic, and preferably soluble in the
propellant. Representative of such agents are the esters or
partial esters of fatty acids containing from 6 to 22 carbon
atoms, such as caproic, octanoic, lauric, palmitic, stearic,
linoleic, linolenic, olesteric and oleic acids with an
15 aliphatic polyhydric alcohol or its cyclic anhydride. Mixed
esters, such as mixed or natural glycerides may be employed.
The surfactant may constitute 0.1%-20% by weight of the
composition, preferably 0.25-5%. the balance of the
composition is ordinarily propellant. A carrier can also be
20 included, as desired, as with, e.g., lecithin for intranasal
delivery.

In addition to the peptides or analogues of the
invention, a pharmaceutical composition may contain suitable
pharmaceutically acceptable carriers, such as excipients,
25 carriers and/or auxiliaries which facilitate processing of
the active compounds into preparations which can be used
pharmaceutically.

The appropriate dosage form will depend on the disease,
the immunogen, and the mode of administration; possibilities
30 include tablets, capsules, lozenges, dental pastes,
suppositories, inhalants, solutions, ointments and parenteral
depots. See, e.g., Berker, supra, Goodman, supra, Avery,
supra and Ebadi, supra, which are entirely incorporated
herein by reference, including all references cited therein.
35 However, it is expected that each vaccine preparation will
include 1-100 μg of the peptide epitope.

The composition may also include an adjuvant. Typical
adjuvants include proteins, peptides, carbohydrates, lipids

and liposaccharides. An example of a currently popular
adjuvant is DETOX (Ribi Immunochemicals)(muramyl dipeptide
and cell wall fragments from Mycobacterium phlei). Other
adjuvants include QS-21, Montanide ISA-21, incomplete

5 Freund's adjuvant, aluminum phosphate, aluminum hydroxide,
alum, DEAE-dextran, saponin, and mineral oil. Montanide ISA-
51 is manufactured by Seppic, Inc. (75 Quai D'Orsay, 75321,
Paris, France). Its composition is manide oleate in mineral
oil solution.

10   QS-21 is manufactured by Cambridge Biotech (365
Plantation Street, Worcester, MA 01605-2376). It is a
triterpene glycoside isolated from the bark of a South
American tree (Quillaja saponaria). The tradename for QS-21
is Stimulon™. Its molecular formula is $C_{92}O_{46}H_{148}$, and its

15 molecular weight is 1,990. Its complete chemical name is
       3-O-β-D-galactopyranosyl-(1->2)-[β-D-xylopyranosyl-(1-
       >3)]-β-D-glucuronpyranosyl-quillaic acid 28-O-β-D-
       apiofuranosyl-(1->3)-β-D-xylopyranosyl-(1->4)-α-L-
       rhamnopyranosyl-(1->2)-3-[5-O-α-L-arabinofuranosyl 3,5-

20   dihydroxy-6-methyloctanoyl]-3,5-dihydroxy-6-
       methyloctanoyl]-β-D-fucopyranoside.
       If desired, the adjuvant may be conjugated to the
epitope and not simply a part of a mixture. See Deres, et
al, Nature, 342:561-4 (1989).

25   The composition may also include an immunomodulator,
especially cytokines such as IL-1, IL-2, IL-4, IL-6, IL-7,
IL-12, Interferon-alpha, Interferon-gamma, Granulocyte
Macrophage Colony Stimulating Factor (GMCSF), Tumor Necrosis
Factor (TNF)-alpha, and TNF- beta.

30   The composition may also include antigen-presenting
cells, such as dendritic cells or macrophages. Preferably,
the APCs are harvested, e.g., from peripheral blood or bone
marrow, conjugated, covalently or noncovalently (e.g., by
pulsing) to the immunogen, e.g., a peptide, and administered

35 to the patient.
       The composition may also include a molecule which
activates or helps in activating CTLs, such as a CD-28
stimulatory molecule (e.g., B7.1, B7.2, or anti-CD28). If

the molecule may be administered in place of the molecule itself.

CD80 (B7 BB1) is expressed on activated B cells and dendritic cells. It is a ligand for CD28 and CTLA-4. It has
5 been found to represent two (partially homologous) proteins, B7-1 and B7-2. See Ramarathinam, et al. T cell costimulation by B7/BB1 induces CD8 T-cell-dependent tumor rejection: an important role of B7/BB1 in the induction, recruitment, and effector function of antitumor T cells. J.Exp. Med. 1994:
10 1790: 1205-1214; Freeman et al. Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. Science 1993, 262: 909-911; Li et al. Costimulation of tumor-reactive CD4+ and CD8+ T lymphocytes by B7, a natural ligand for CD28, can be used to treat
15 established mouse melanoma. J. Immunol. 1994, 153: 421-428; Hodge et al. Admixture of a recombinant vaccinia virus containing the gene for the costimulator molecule B7 and a recombinant vaccinia virus containing a tumor-associated antigen gene results in enhanced specific T-cell responses
20 antitumor immunity. Cancer Res. 1995, 55: 3598-3603.

A pharmaceutical composition according to the present invention may further comprise at least one cancer chemo- therapeutic compound, such as one selected from the group consisting of an anti-metabolite, a bleomycin peptide
25 antibiotic, a podophyllin alkaloid, a *Vinca* alkaloid, an alkylating agent, an antibiotic, cisplatin, or a nitrosourea. A pharmaceutical composition according to the present invention may further or additionally comprise at least one viral chemotherapeutic compound selected from gamma globulin,
30 amantadine, guanidine, hydroxybenzimidazole, interferon-α, interferon-β, interferon-γ, thiosemicarbarzones, methisazone, rifampin, ribvirin, a pyrimidine analog, a purine analog, foscarnet, phosphonoacetic acid, acyclovir, dideoxy- nucleosides, or ganciclovir. See, e.g., Katzung, *supra*, and
35 the references cited therein on pages 798-800 and 680-681, respectively, which references are herein entirely incorporated by reference.

As an alternative to a pharmaceutical composition

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comprising the immunogen of the present invention, per se, the pharmaceutical composition may instead comprise a vector comprising an expressible gene encoding such an immunogen. The pharmaceutical composition and method would then be

5   chosen so that the vector was delivered to suitable cells of the subject, so that the gene would be expressed and the immunogen produced in such a manner as to elicit an immune response. A preferred vector would be a Vaccinia virus, such as a construct containing a minigene encoding the peptide of

10 interest. In the case of genes encoding naturally occurring proteins, or peptide fragments thereof, one may, but need not, use the DNA sequence which encodes the proteins or peptides in nature. A preferred route for immunization would be scarification. A preferred immunization protocol would be

15 10E6 to 10E8 pfu/dose in the initial injection, followed up with boosters at 1,3 and 12 months. The boosters could be the previously described immunogen-containing composition.

In the case of genes encoding naturally occurring proteins, or peptide fragments thereof, one may, but need

20 not, use the DNA sequence which encodes the proteins or peptides in nature.

Recombinant vaccinia virus constructs have been used for immunization against hepatitis B (Moss, et al., Nature, 311, 67, 1984), herpes simplex virus (Wacchsman, et al., Biosci.

25 Rep. 8, 323; 334, 1988), parainfluenza type 3 (Spriggs, et al., J. Virol., 62, 1293, 1988), and Lassa fever virus (Fisher-Hoch, et al., Proc. Natl. Acad. Sci. USA, 86, 317, 1989). Vaccinia virus constructs comprising gene for cancer-associated antigens have also been prepared (Lathe, et al.,

30 Nature, 326, 878, 1987; Bernards, et al., Proc. Natl. Acad. Sci. USA, 84, 6854, 1987; Estin, et al., Proc. Natl. Acad. Sci. USA, 85, 1052, 1988).

Alternatively or additionally, the composition may comprise disease-specific CTL. Antigenic peptides may be

35 used to elicit CTL ex vivo. Ex vivo CTL responses to an antigen are induced by incubating in tissue culture the patient's CTL precursor cells (CTLp) together with a source of antigen-presenting cells (APC) and the appropriate

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immunogenic peptide. After an appropriate incubation time
(typically 1-8 weeks), in which the CTLp are activated and
mature and expand into effector CTL, the cells are infused
back into the patient, where they will destroy their specific
5 target cell. In order to optimize the *in vitro* conditions
for the generation of specific cytotoxic T cells, the culture
of stimulator cells may be maintained in an appropriate
serum-free medium.

    Prior to incubation of the stimulator cells with the
10 cells to be activated, e.g., precursor CD8+ cells, an amount
of antigenic peptide is added to the stimulator cell culture,
of sufficient quantity to become loaded onto the human Class
I molecules to be expressed on the surface of the stimulator
cells. In the present invention, a sufficient amount of
15 peptide is an amount that will allow about 200, and
preferably 200 or more, human Class I MHC molecules loaded
with peptide to be expressed on the surface of each
stimulator cell. Preferably, the stimulator cells are
incubated with at least 1 mg/ml, more preferably >20μg/ml
20 peptide.

    Resting or precursor CD8+ cells are then incubated in
culture with the appropriate stimulator cells for a time
period sufficient to activate the CD8+ cells. Preferably,
the CD8+ cells are activated in an antigen-specific manner.
25 The ratio of resting or precursor CD8+ (effector) cells to
stimulator cells may vary from individual to individual and
may further depend upon variables such as the amenability of
an individual's lymphocytes to culturing conditions and the
nature and severity of the disease condition or other
30 condition for which the within-described treatment modality
is used. Preferably, however, the lymphocyte:stimulator cell
ratio is in the range of about 1:5 to 20:1, more preferably
3:1 to 5:1. The effector/stimulator culture may be
maintained for as long a time as is necessary to stimulate a
35 therapeutically useful or effective number of CD8+ cells.

    The induction of CTL *in vitro* requires the specific
recognition of peptides that are bound to allele specific MHC
class I molecules on APC. The number of specific MHC/peptide

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complexes per APC is crucial for the stimulation of CTL, particularly in primary immune responses. While small amounts of peptide/MHC complexes per cell are sufficient to render a cell susceptible to lysis by CTL, or to stimulate a

5 secondary CTL response, the successful activation of a CTL precursor (pCTL) during primary response requires a significantly higher number of MHC/peptide complexes. Peptide loading of empty major histocompatability complex molecules on cells allows the induction of primary cytotoxic

10 T lymphocyte responses.

Often it is useful, in the generation of peptide-specific CTL, to stimulate with mutant cell lines that have some empty MHC molecules. An exmample is the human lymphoid cell line, T2. However, mutant cell lines expressing every

15 MHC molecule are not yet available. Thus, in some cases, it may be useful to strip endogenous MHC-associated peptides from the surface of APC, followed by loading the resulting empty MHC molecules with the immunogenic peptides of interest. The use of non-transformed (non-tumorigenic), non-

20 infected cells, and preferably, autologous cells of patients as APC is desirable for the design of CTL induction protocols directed towards development of *ex vivo* CTL therapies. This application discloses methods for stripping the endogenous MHC-associated peptides from the surface of APC followed by

25 the loading of desired peptides.

A stable MHC class I molecule is a trimeric complex formed of the following elements: 1) a peptide usually of 8 - 10 residues, 2) a transmembrane heavy polymorphic protein chain which bears the peptide-binding site in its $\alpha 1$ and $\alpha 2$

30 domains, and 3) a non-covalently associated non-polymorphic light chain, $\beta_2$microglobulin. Removing the bound peptides and/or dissociating the $\beta_2$microglobulin from the complex renders the MHC class I molecules nonfunctional and unstable, resulting in rapid degradation. All MHC class I molecules

35 isolated from PBMCs have endogenous peptides bound to them. Therefore, the first step is to remove all endogenous peptides bound to MHC class I molecules on the APC without causing their degradation before exogenous peptides can be

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added to them.

Two possible ways to free up MHC class I molecules of
bound peptides include the culture temperature from 37°C to
26°C overnight to destabilize $\beta_2$microglobulin and stripping
5   the endogenous peptides from the cell using a mild acid
treatment. The methods release previously bound peptides
into the extracellular environment allowing new exogenous
peptides to bind to the empty class I molecules. The cold-
temperature incubation method enables exogenous peptides to
10   bind efficiently to the MHC complex, but requires an
overnight incubation at 26°C which may slow the cell's
metabolic rate. It is also likely that cells not actively
synthesizing MHC molecules (e.g., resting PBMC) would not
produce high amounts of empty surface MHC molecules by the
15   cold temperature procedure.

Harsh acid stripping involves extraction of the peptides
with trifluoroacetic acid, pH 2, or acid denaturation of the
immunoaffinity purified class I-peptide complexes. These
methods are not feasible for CTL induction, since it is
20   important to remove the endogenous peptides while preserving
APC viability and an optimal metabolic state which is
critical for antigen presentation. Mild acid solutions of pH
3 such as glycine or citrate-phosphate buffers have been used
to identify endogenous peptides and to identify tumor
25   associated T cell epitopes. The treatment is especially
effective, in that only the MHC class I molecules are
destabilized (and associated peptides released), while other
surface antigens remain intact, including MHC class II
molecules. Most importantly, treatment of cells with the
30   mild acid solutions do not affect the cell's viability or
metabolic state. The mild acid treatment is rapid since the
stripping of the endogenous peptides occurs in two minutes at
4°C and the APC is ready to perform its function after the
appropriate peptides are loaded. The technique is utilized
35   herein to make peptide-specific APCs for the generation of
primary antigen-specific CTL. The resulting APC are
efficient in inducing peptide-specific CD8+ CTL.

Activated CD8+ cells may be effectively separated from

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the stimulator cells using one of a variety of known methods.
For example, monoclonal antibodies specific for the
stimulator cells, for the peptides loaded onto the stimulator
cells, or for the CD8+ cells (or a segment thereof) may be

5   utilized to bind their appropriate complementary ligand.
Antibody-tagged molecules may then be extracted from the
stimulator-effector cell admixture via appropriate means,
e.g., via well-known immunoprecipitation or immunoassay
methods.

10   Effective, cytotoxic amounts of the activated CD8+ cells
can vary between *in vitro* and *in vivo* uses, as well as with
the amount and type of cells that are the ultimate target of
these killer cells. The amount will also vary depending on
the condition of the patient and should be determined via

15   consideration of all appropriate factors by the practitioner.
Preferably, however, about 1×10^6 to about 1×10^{12}, more
preferably about 1×10^8 to about 1×10^{11}, and even more
preferably, about 1×10^9 to about 1×10^{10} activated CD8+
cells are utilized for adult humans, compared to about 5×10^6

20   - 5×10^7 cells are used in mice.

         Preferably, as discussed above, the activated CD8+ cells
are harvested from the cell culture prior to administration
of the CD8+ cells to the individual being treated. It is
important to note, however, that unlike other present and

25   proposed treatment modalities, the present method preferably
uses a cell culture system that is not tumorigenic.
Therefore, if complete separation of stimulator cells and
activated CD8+ cells is not achieved, there is no inherent
danger known to be associated with the administration of a

30   small number of stimulator cells, whereas administration of
mammalian tumor-promoting cells may be extremely hazardous.

         Methods of re-introducing cellular components are known
in the art and include procedures such as those exemplified
in U.S. Patent No. 4,844,893 to Honsik, et al. and U.S.

35   Patent No. 4,690,915 to Rosenberg. For example,
administration of activated CD8+ cells via intravenous
infusion is appropriate.

         Adoptive transfer of melanoma-specific CTL has been

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accompanied by tumor shrinkage in a large minority of
patients with advanced melanoma and by disappearance of all
detectable tumor in a smaller proportion of patients.
(Rosenberg et al, NEM 319: 1676-1680, 1988) and in animal

5   studies appears to be particularly promising for the treat-
ment of solid tumors (Rosenberg SA et al. Science 233:1318-
1321). One of the problems with existing methods for CTL
generation is that they require the resection of large
metastic tumor deposits to initiate the process. If the

10   requirement for available autologous tumor could be
circumvented, then patients with no measurable disease but a
high risk of recurrence (eg, melanoma patients with primary
tumors greater than 4 mm thick or with microscopic tumor
metastatic to regional nodes) could be treated with adoptive

15   therapy even if their tumor were removed and fixed in
formalin and no other gross tumor was evident. These
patients have a very high likelihood of harboring
micrometastic disease for which no other effective therapy is
now available; so most will die of the melanoma. It is

20   possible that the presence of bulky tumor suppresses the
autologous immune response; so treatment of patients without
bulky disease would be an attractive goal. Especially in
murine systems, CTL have been generated and maintained by
stimulation with cells to which the peptide epitope has been

25   bound. We propose that, e.g., HLA-A2.1+ or HLA-A3+ cells
(autologous B cells, macrophages, or dendritic cells,
ideally), would be pulsed in vitro with peptide (e.g., a
cysteine-depleted form of peptide KCDICTDEY) and used as in
vitro simulators for autologous lymph node cells or

30   peripheral blood lymphocytes. The patients could be pre-
stimulated with a peptide vaccine prior lymphocyte harvest if
the existing response was inadequate. Lymphocytes stimulated
with peptide in vitro could then be expanded to 10^{10} or 10^{11}
cells, then re-infused into the patients in a manner

35   analogous to that used for LAK cell therapy. It is expected
that the adoptively transferred CTL would survive best with
IL-2 infusion at low to intermediate doses, and that putative
inhibitors of Tc suppression (eg: cyclophosphamide) may be

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employed also, prior to the infusions of CTL.

Clinical studies with adoptive immunotherapy using A2-restricted tumor infiltrating lymphocytes (TIL) have shown a strong correlation between Pmel-17/gp100 reactivity and
5   positive clinical responses of patients treated with those TIL. Kawakami, et al., J. Immunol., 154:3961-8 (1995).

Disease-Specific Diagnostic Agents

A melanoma-specific diagnostic agent is (1) a molecule which is or which comprises a disease-specific epitope as
10   previously defined, and which is labeled, immobilized, or otherwise rendered suitable for diagnostic use, or (2) an antibody which specifically binds such a disease-specific epitope, and which is labeled, immobilized, or otherwise rendered suitable for diagnostic use, or (3) a T-cell line
15   (e.g., murine or human), which specifically recognizes a disease-specific epitope.

Diagnostic Uses and Compositions

The relationship between the host's immune response and his or her tumor is poorly understood. Better understanding
20   of that response depends on evaluation of the specific responses against individual epitopes. If patients do have an immune response to an epitope naturally, then evaluation and quantitation of that by precursor frequency analysis of the CTL in the patient's blood pool may permit some
25   assessment of the protection that person's immune system is providing. As new therapies become available for melanoma, it may be useful to screen patients for the presence of the peptide on their tumor and the presence of CTL in their blood pool with specificity for the peptide on appropriate cells.
30   These findings may determine whether further augmentation of the immune response is indicated or whether other, non-immunologic, therapy should be employed. A parallel to this is the determination on breast cancers of the presence of estrogen and progesterone receptors before considering
35   hormonal therapy or chemotherapy.

Thus, the peptides of the present invention may be used

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to screen a sample for the presence of an antigen with the
same epitope, or with a different but cross-reactive epitope,
or for the presence of CTLs which specifically recognize the
corresponding epitopes. The sample will normally be a
5 biological fluid, such as blood, urine, lymphatic fluid,
amniotic fluid, semen, saliva, tears, milk, or cerebrospinal
fluid, or a fraction or derivative thereof, or a biological
tissue, in the form of, e.g., a tissue section or homogenate.
The preferred sample is blood, or a fraction or derivative
10 thereof.

Assays may be divided into two basic types, hetero-
geneous and homogeneous. In heterogeneous assays, the
interaction between the affinity molecule and the analyte
does not affect the label, hence, to determine the amount or
15 presence of analyte, bound label must be separated from free
label. In homogeneous assays, the interaction does affect
the activity of the label, and therefore analyte levels can
be deduced without the need for a separation step.

Assays may also be divided into competitive and non-
20 competitive formats. In the competitive format, the analyte
competes with a labeled analyte analogue for binding to a
binding partner. In a common noncompetitive format called a
sandwich assay, the analyte is first bound by a capture
reagent, and then by a tag reagent.

25 In order to detect the presence, or measure the amount,
of an analyte, the assay must provide for a signal producing
system (SPS) in which there is a detectable difference in the
signal produced, depending on whether the analyte is present
or absent (or, in a quantitative assay, on the amount of the
30 analyte). The detectable signal may be one which is visually
detectable, or one detectable only with instruments.
Possible signals include production of colored or luminescent
products, alteration of the characteristics (including
amplitude or polarization) of absorption or emission of
35 radiation by an assay component or product, and precipitation
or agglutination of a component or product. The term
"signal" is intended to include the discontinuance of an
existing signal, or a change in the rate of change of an

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observable parameter, rather than a change in its absolute
value. The signal may be monitored manually or automatically.

The component of the signal producing system which is
most intimately associated with the diagnostic reagent is
5   called the "label". A label may be, e.g., a radioisotope, a
fluorophore, an enzyme, a co-enzyme, an enzyme substrate, an
electron-dense compound, an agglutinable particle.

The radioactive isotope can be detected by such means as
the use of a gamma counter or a scintillation counter or by
10 autoradiography. Isotopes which are particularly useful for
the purpose of the present invention are ^3H, ^{125}I, ^{131}I, ^{35}S, ^{14}C,
and, preferably, ^{125}I.

It is also possible to label a compound with a
fluorescent compound. When the fluorescently labeled anti-
15 body is exposed to light of the proper wave length, its
presence can then be detected due to fluorescence. Among the
most commonly used fluorescent labelling compounds are
fluorescein isothiocyanate, rhodamine, phycoerythrin,
phycocyanin, allophycocyanin, o-phthaldehyde and
20 fluorescamine.

Alternatively, fluorescence-emitting metals such as
^{125}Eu, or others of the lanthanide series, may be attached to
the binding protein using such metal chelating groups as
diethyl-enetriaminepentaacetic acid (DTPA) and
25 ethylenediamine-tetraacetic acid (EDTA).

The peptides also can be detectably labeled by coupling
to a chemiluminescent compound. The presence of the chem-
iluminescently labeled antibody is then determined by
detecting the presence of luminescence that arises during the
30 course of a chemical reaction. Examples of particularly
useful chemiluminescent labeling compounds are luminol,
isolumino, theromatic acridinium ester, imidazole, acridinium
salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label
35 the peptides. Bioluminescence is a type of chemiluminescence
found in biological systems in which a catalytic protein
increases the efficiency of the chemiluminescent reaction.
The presence of a bioluminescent protein is determined by

detecting the presence of luminescence. Important bio-luminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Enzyme labels, such as horseradish peroxidase, alkaline
5   phosphatase, malate dehydrogenase, staphylococcal nuclease, δ-V-steroid isomerase, yeast alcohol dehydrogenase, α-glycero phosphate dehydrogenase, triose phosphate isomerase, asparaginase, glucose oxidase, β-galactosidase, ribonuclease, glucose-6-phosphate dehydrogenase, glucoamylase and acetyl-
10 choline esterase, are preferred. When an enzyme label is used, the signal producing system must also include a substrate for the enzyme. If the enzymatic reaction product is not itself detectable, the SPS will include one or more additional reactants so that a detectable product appears.

15 A label may be conjugated, directly or indirectly (e.g., through a labeled antibody), covalently (e.g., with SPDP) or noncovalently, to the peptide, to produce a diagnostic reagent. Similarly, the peptide may be conjugated to a solid phase support to form a solid phase ("capture") diagnostic
20 reagent. Suitable supports include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the
25 present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to its target. Thus the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the
30 external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc.

Additionally, the peptides may be used as a diagnostic tool to evaluate whether other immunotherapeutic treatments (tumor vaccines of any kind, adoptive transfer of CTL, etc)
35 are having a beneficial effect.

Generation of tumor-specific cytotoxic T cells

Detailed methods of CTL generation have been previously reported. Malignant melanoma metastatic to cervical lymph

nodes was resected from an 80-yr old patient designated VMM5.
The nodes were mechanically dissociated and then
enzymatically digested in Eagle's MEM (GIBCO, Grand Island,
NY) containing 2.5% FCS (GIBCO, or Whittaker, Walkersville,
5   MD), 0.1% collagenase B (Boehringer-Mannheim, Indianapolis,
IN), 0.002% DNase (Sigma, St. Louis, MO), penicillin 100
U/ml, streptomycin 100 microg/ml (Pen- Strept, GIBCO) at room
temperature. After 4 h, dissociated cells were collected and
cryopreserved. Remaining tumor fragments were returned to
10 the digestion media overnight. The digests were harvested in
a similar fashion daily for 3 days, with viable tumor cells
and lymphocytes obtained each day. These cells were
cryopreserved in FCS + 10% DMSO in liquid nitrogen. Initial
cultures were established with the mixture of lymphocytes and
15 tumor from the tumor-involved node. The ratio of tumor cells
to lymphocytes placed in culture were approximately 1:1. The
cells were cultured in 24-well tissue culture plates (Linbro,
Hamden, CT) in RPMI 1640 (Sigma) containing 10% FCS, Pen-
Strept, and 20 U/ml rIL-2 (Cetus, Emeryville, CA). The CTL
20 were restimulated with irradiated (100 Gy) fresh cryo-
preserved autologous tumor (VMM5) at a TLR (tumor:lymphocyte
ratio) of 1:10 on day 16. Beginning with the third in vitro
stimulation (day 32), and thereafter every 10 to 15 days, the
CTL were restimulated with the allogeneic HLA-A2.1⁺ melanoma
25 cell line DM6. A TLR of 1:5 was used until the cells were 70
days old, after which a TLR between 1:2 and 2:1 was used.
Several VMM5 CTL lines were generated following this protocol
closely and with consistent results from each. Similar
methods were enployed for generation of other CTL lines
30 studied.

Cytotoxicity Assays

      Cell-mediated killing was determined in vitro using a 4-
h chromium release assay. 51Cr-labeled target cells were
plated at 2×10^3 cells/well in triplicate on 96-well V-bottom
35 plates (Costar, Cambridge, MA) with varying numbers of
effector cells in a final volume of 250 microl. Wells
containing either culture medium and target cells only or 1 M

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HCl and target cells served as background 51Cr release
controls and total release controls, respectively. The
plates were centrifuged at 100 x g for 3 min and incubated at
37°C in 5% CO2 for 4 h. The plates were again centrifuged,

5 and 0.20 ml of medium from each well was removed for counting
in a gamma counter. The cytotoxic index was calculated as:

$$\frac{Cpm\ (experimental) - cpm\ (background)}{Cpm\ (total\ release) - cpm\ (background)} \times 100\%$$

10 Lytic units were calculated for several of the cytotoxicity
assays, using a software package prepared by the National
Cancer Institute (Bethesda, MD), which solves for the
equation y = A x [1 - exp(-kx)], where x is the E:T ratio, y
is the cytotoxic index, A is the curve maximum, and k is a

15 constant used to calculate the slope of the best fit line.
For the purposes of this study, a lytic unit was defined as
the number of effector cells needed to mediate 30% lysis of
target cells. The number of lytic units was calculated per
10^5 effector cells (LU30 per 10^5 cells).

20 <u>Prior Examples</u>
        The identification of melanoma-specific HLA-A2 and HLA-
A3 epitopes is described in WO95/22561 and PCT/US97/04958,
both incorporated by reference herein.

EXAMPLE A1-1
25 **Identification of a Tyrosinase Epitope Recognized by Human
Melanoma-Reactive, HLA-A1 Restricted CTLs.**
<u>Introduction</u>
        We have identified the peptide KCDICTDEY (K is N-
terminal), from the tyrosinase protein, as an epitope for
30 HLA-A1-restricted melanoma-specific cytotoxic T-lymphocytes
(CTL). This work has been done by generating HLA-A1-
restricted melanoma-reactive CTL, creating a vaccinia
construct encoding the intact human tyrosinase gene, then
infecting HLA-A1+ non-melanoma target cells with the vac-
35 tyrosinase construct. In doing so, VMM12 CTL and VMM15 CTL
both recognize an HLA-A1-associated peptide derived from

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tyrosinase. We have since screened a large panel of peptides
that we predicted to bind to HLA-A1, from the defined
sequence of tyrosinase. The peptide KCDICTDEY, when pulsed
onto HLA-A1+ non-melanoma cells (C1R-A1), reconstitutes an
5   epitope for VMM15 CTL. To a lesser extent, two other
peptides that are longer than 9-residues, but which contain
the entire KCDICTDEY sequence, also reconstitute an epitope
for these CTL. None of 116 other peptides tested worked.
Thus, we believe this is an epitope which can be used as an
10 immunogen in treating or preventing melanoma in the 20-25% of
patients who express HLA-A1.
     Cell lines and HLA typing: The human melanoma cell lines
VMM1, VMM12, VMM15, VMM18, VMM30 and VMM34 were derived from
patients at the University of Virginia (Charlottesville, VA).
15 Other fresh (uncultured) tumors VMM14, VMM21 and VMM40 were
also prepared from surgical specimens from patients at the
University of Virginia. DM6 and DM331 were provided by Drs.
H.F. Seigler and T.L. Darrow at Duke University (Durham, NC).
Immunohistochemical staining of these cell lines with S-100,
20 HMB-45 and vimentin antibodies was characteristic of
melanoma, while staining for epithelial membrane antigen and
cytokeratin was negative. The Na8Mel and Na8Mel+Tyr cell
lines were a gift from Vincent Brichard and Thierry Boon.
Na8Mel is a tyrosinase-negative human melanoma line, and
25 Na8Mel+Tyr was derived from transfection of Na8Mel with the
tyrosinase cDNA 123.B2. The human melanoma line SkMel24 was
obtained from the American Type Culture Collection. The CV-1
and 143B TK- lines used in the propagation of vaccinia virus
were also obtained from the American Type Culture Collection
30 (ATCC, Bethesda, MD). VMM12-Epstein-Barr-virus-(EBV)-
transformed B lymphoblastoids cell lines (B-LCL) were
generated from peripheral blood mononuclear cells (PBMC) of
melanoma patient VMM12. Briefly, the PBMC were incubated with
filtered supernatant from the EBV producing cell line B-958
35 for 1 h at 37oC, followed by culture in RPMI 1640 media with
10% fetal calf serum (FCS) and antibiotics, plus a 1:100
dilution of PHA. K562 is an NK-sensitive human
erythroleukemia line. T2-A3 (an HLA-A3 transfectant of the

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antigen-processing-defective mutant human lymphoid cell line, T2) was provided by P. Cresswell. HLA typing was performed by microcytotoxicity assay on autologous lymphocytes (Gentrak). The HLA class I transfectants of the C1R cell line (C1R-A1,

5   C1R-A3, C1R-B7, and C1R-B8) were kindly provided by Dr. Peter Cresswell (Yale University, New Haven, CT). Expression of HLA-A1 and -A3 by tumor cells was confirmed by staining with the monoclonal antibodies HA-A1 (One Lambda, Canoga Park, CA) and GAP-A3 (kindly provided by P. Cresswell). Melanoma

10   reactive CTL from peripheral blood lymphocytes, from tumor-involved nodes, or from tumor-draining nodes were generated in vitro by repeated stimulation with autologous tumor cells. Tissue culture media used for the human cell lines was RPMI-1640 (Sigma) supplemented with 10% fetal calf serum,

15   glutamine, and antibiotics, hereafter referred to simply as RPMI.
     CTL lines: We have generated human melanoma-specific CTL lines by in vitro stimulation with autologous tumor, from patients whose tumors express melanocytic tissue

20   differentiation antigens and express one or more of the MHC molecules A1, A3, B7, and B8. Methods for CTL generation have been described.
     Production of recombinant vaccinia virus expressing the human genes encoding melanocytic tissue differentiation antigens:

25   We have examined class I MHC-associated epitopes for the melanocytic tissue differentiation antigens by using vaccinia constructs for each of the genes Pmel17/gp100, tyrosinase, and MART-1/MelanA. A cDNA clone of the Pmel17 gene (HUMPMEL17 - Genbank) was generously provided by S.N. Wagner,

30   Essen, Germany. The tyrosinase gene was provided by Thierry Boon, Brussels. We have PCR cloned out a cDNA clone of the MART-1/Melan-A gene from DM6 melanoma cells. The entire open-reading frame for each of these cDNA's was sub-cloned into a modified pSC11 vector (Ref Hahn JEM 1991) adjacent to

35   the vaccinia P7.5 early/late promoter using standard recombinant DNA methods. Standard dideoxy sequencing was used to confirm insertion and orientation. A recombinant vaccinia virus expressing the protein encoded by this gene (vac-Pmel-

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17) was generated using published methods (Ref Macket J.Virol 1984). Briefly, CV-1 cells were infected with the parental WR strain of vaccinia virus and transfected (Lipofectin, Gibco-BRL) with the pSC11.3-Pmel-17 plasmid. Thymidine-kinase

5   negative recombinants were amplified in 143B TK⁻ cells in the presence of bromodeoxyuridine (Sigma). Recombinants with beta-galactosidase activity were isolated and cloned through several rounds of plaque purification. Large-scale stocks were produced, sucrose purified, and titered in CV-1 cells.

10   The resulting recombinant vaccinia viruses were used to infect the lymphoblastoid cell lines C1R-A1, C1R-A2, C1R-A3, C1R-B7, and C1R-B8, where C1R is a human lymphoblastoid line devoid of native expression of HLA-A or HLA-B region molecules, but expressing low levels of HLA-C and MHC Class

15   II molecules. In some cases EBV-transformed B cells with defined MHC expression were used for the infections. This resulted in transient expression of the antigens of interest. These cells were assayed for recognition by CTL in Cr51-release assays. As a negative control, target cells were

20   also infected with a recombinant vaccinia virus with an irrelevant DNA insert (influenza nucleoprotein, NP). Thus, the cell lines listed above permit isolated evaluation of the expression of antigenic peptides in association with the common Class I MHC molecules HLA-A1, A2, A3, B7, and B8.

25   *Evaluating recognition of target cells by CTL.*
Reactivity was assessed by cytotoxicity in a 4-hour chromium release assay. Transient expression of individual melanoma differentiation antigens in non-melanoma cells was accomplished by infecting target cells with recombinant

30   vaccinia viruses using 50 plaque forming units (titered on CV-1) per cell for 5-7 hours at 37°C prior to ⁵¹Cr-labeling. Peptide-pulsed targets were generated by diluting peptides in RPMI and pre-incubating with ⁵¹Cr -labeled target cells for 2 hours prior to the addition of CTL. Assay wells containing

35   peptide and target cells but no CTL were used as controls to rule out toxicity of the peptides themselves. Positive controls were the autologous tumor and known cross-reactive tumor lines. A negative control was uninfected C1R-MHC line

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and a C1R-MHC line transfected with a vaccinia construct
expressing influenza nucleoprotein, vac-NP only. Briefly,
^{51}Cr-labeled target cells were plated at 1 - 2x10^3 cells/well
in triplicate on 96-well V-bottom plates (Costar, Cambridge,
5   MA) with indicated ratio of effector cells in a final volume
of 200 microliters. Wells containing either culture medium or
1M HCl in place of the effector cells served as spontaneous
and maximum ^{51}Cr-release controls, respectively. Plates were
centrifuged at 100 x g for 3 min and incubated at 37 °C for 4
10   h, after which 150 microliters of supernatant from each well
was counted on a gamma counter (ICN). The percent specific
lysis was calculated using the equation: [(experimental
release - spontaneous release) / (maximum release -
spontaneous release)] x 100. Vaccinia infected targets were
15   generated by infecting cells with 50 pfu/cell of appropriate
recombinant vaccinia virus at 37°C for 5 h, prior to ^{51}Cr-
labeling.

Peptide synthesis and Reconstitution with synthetic peptides:
Peptide sequences were selected from the reported human
20   sequence of tyrosinase, based on predicted HLA-A1 binding
motifs (see table 10). These peptides were synthesized by
standard Fmoc chemistry using a Gilson model AMS422 peptide
synthesizer. Biologically active peptides identified at
initial screening were purified to >98% by reversed-phase
25   HPLC on a Vydac C-4 column with 0.05% trifluoroacetic acid
(TFA):water and an acetonitrile gradient and then re-
evaluated in cytolysis assays. Peptides were reconstituted
in CTL assay medium (RPMI 1640, 10% FCS, antibiotics) and
pre-incubated for 2 h with 2x10^3 ^{51}Cr labeled target cells in
30   100 microliters/well in 96-well plates. Effector cells were
added in 100 microliters assay medium for a final effector to
target (E:T) ratio of 20:1 and the remainder of the assay was
performed as in standard chromium release assays described
above. Wells containing peptide and target cells but no CTL
35   were used as controls to rule out toxicity of the peptides
themselves. Initial experiments were performed with
unpurified synthetic peptides. Biologically active peptides
identified at initial screening were then purified to >98% by

reversed-phase HPLC on a Vydac C-4 column with 0.05%
trifluoroacetic acid:water and an acetonitrile gradient, then
re-evaluated in CTL assays.

Results

5   Melanoma-reactive CTL lines recognize MHC-associated peptides
from several melanocytic differentiation antigens

The CTL lines listed in Table 111 were evaluated for
recognition of peptides derived from the 3 melanocytic tissue
differentiation antigens listed above, in chromium-release
10 assays, by transient infection with vaccinia constructs
encoding those genes. Examples of their reactivity against
HLA-matched allogeneic melanomas are shown in Figure 1. A
summary of these results with vaccinia constructs are listed
in Table 112 and are shown in Figure 2. Responses to
15 tyrosinase peptides were observed in half of cases. In
addition, responses to MART-1 and gp100 peptides were
observed in a smaller set of CTL lines.
At least two of the HLA-A1+ CTL lines recognized tyrosinase
peptides in an HLA-A1-restricted manner.

20   VMM12 CTL and VMM15 CTL were assayed initially on
autologous EBV-B cells as targets. Reactivity against
tyrosinase was observed, so additional studies were performed
to confirm the reactivity and to determine the MHC
restriction (Figure 2). C1R cells that express selected
25 Class I MHC molecules only were used as target cells. As
seen in Figure 2, C1R-A1 cells infected with vac-tyrosinase
are recognized by VMM12 and VMM15 CTL, confirming that one or
more tyrosinase-derived peptides are recognized by VMM12 and
VMM15 CTL in association with HLA-A1.

30 The peptide representing residues 243-251 of tyrosinase
reconstitutes an epitope for VMM15 CTL.
A set of peptides were synthesized from the defined amino
acid sequence of tyrosinase, including 9-mers and longer
peptides, with tyrosine (Y) at the C-terminal position and T,
35 S, or M at position 2 and/or D, E, A, or S at position 3.
These were assayed for their ability to reconstitute epitopes
for melanoma-reactive CTL VMM12 and VMM15. C1R-A1 cells were
pulsed with the peptide at concentrations ranging from 0.1 to

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10 uM in normal assay medium (RPMI + 10% FCS), then evaluated
for recognition in a chromium-release assay. As shown in
Figure 3, three peptides were recognized by VMM15 CTL, all
containing the sequence KCDICTDEY (tyrosinase residues 243-
5   251). The most effective, even at the lowest concentration
tested, was the 9-mer peptide KCDICTDEY, but also recognized
were a ten-mer, EKCDICTDEY, and a 13-mer, DAEKCDICTDEY
(Figure 3).

       Similar reactivity was seen with VMM12 CTL as well,
10 suggesting that KCDICTDEY is a shared antigen on human
melanoma cells expressing HLA-A1, against which multiple
patients' CTL may be expected to react (Figure 4). The
location of this peptide in the intact protein tyrosinase is
shown in Figure 5.

15   **Discussion**
       The peptide KCDICTDEY appears to be recognized by CTL
from at least two different patients, in association with
HLA-A1. Although longer peptides also are reactive, the
dominant response seems to be to KCDICTDEY. This peptide is
20 unusual in its large number of polar amino acid residues,
including two aspartic acid residues, one glutamic acid
residue, and two cystine residues. The tyrosine residue at
position 9 and the aspartic acid at position 3 are important
for binding to the MHC.   By a computerized system for
25 predicting the binding affinity of individual peptides to
HLA-A1 (and other HLA haplotypes), see
http://bimas.dcrt.nih.gov:80/cgi-
bin/molbio/ken_parker_comboform (The algorithm for this
software is discussed in Parker, et al., J. Immunol., 152:163
30 (1994)), this peptide is predicted to the be the tyrosinase
peptide with highest affinity for HLA-A1, which may make it
useful for immunization after pulsing on antigen-presenting
cells.
       KCDICTDEY is associated with half-maximal lysis at
35 approximately 1 ug/ml (1 uM). The potency of this activity
may be increased by various amino acid substitutions as
taught above.

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There have been two peptides described as epitopes for melanoma-reactive HLA-A1-restricted CTL. They are the MAGE-1 and MAGE-3 peptides EADPTGHSY and EVDPIGHLY· While these have substantial potential value as immunogens, only a subset of
5   melanoma patients express them. Most other MHC-associated peptide epitopes are HLA-A2 associated. However, HLA-A1 is expressed in approximately 29% of patients in this country. We have previously described an HLA-A3-associated epitope from gp100, ALLAVGATK. Now, with defined peptide epitopes
10   known, it is possible to consider the use of a multivalent peptide vaccine, where all patients expressing either HLA-A1, HLA-A2, or HLA-A3, which is approximately 70% of the patients at risk, may be treated with specific vaccine therapy.

Table 111. CTL lines studied for recognition of target cells infected with vaccinia constructs encoding Pmel17/gp100, Tyrosinase, or MART-1/MelanA

Melanoma Patient ID	Class I MHC expression	Target cell	MHC shared with target	Pmel17-reactive	Tyrosinase-reactive	MART1-reactive
VMM12	A1, A3, B7, B14	VMM12-EBV	A1, A3, B7, B14	0	Yes	---
		VMM15-EBV	A1	0	Yes	0
		C1R-A1	A1	---	Yes	---
		C1R-A3	A3	---	0	---
		C1R-B7	B7	---	0	---
VMM15	A1, A25, B8, B18	VMM15-EBV	A1, A25, B8, B18	0	Yes	Yes
		C1R-A1	A1	---	Yes	---
		C1R-B8	B8	---	+/-	---
		VMM38-EBV	B18	---	0	---
VMM10	A3, A25, B62, C1, C4	VMM15-EBV	A25	0	+/-	+/-
		VMM12-EBV	A3	0	0	0
		VMM16-EBV	C1, C4	0	0	0
VMM30	A1, A2, B27, B57, C2, C6	VMM30-EBV	A1, A2, B27, B57, C3	0	Yes	0
VMM14	A1, A25, B8, B48	VMM15-EBV	A1, A25, B8	0	Yes	0
VMM21	A1, A2, B7, B37	VMM21-EBV	A1, A2, B7, B37	0	0	Yes
VMM18	A3, A31/33, B60, C3	VMM18-EBV	A3, A31/33, B60, C3	Yes	0	Yes
		VMM12-EBV	A3	Yes	0	---
		C1R-A3	A3	Yes	---	Yes
		VMM17-EBV	A33?	0	---	0

VMM19	A24, B35, B55	VMM19-EBV	A24, B35, B55	+/-	0	0
DM331	A1, A2, B15, B62	VMM12-EBV	A1, A2, B15, B62	0	0	---
VMM39	A2, A3, B7, B44	VMM12-EBV	A3, B7	0	0	0
		VMM30-EBV	A2	0	0	+/-

--- = not tested
5 +/- = results are equivocal and need further investigation.

Table 112.   Summary of CTL reactivities observed

Patient ID	Source of CTL epitope	Restricting Class I MHC molecule
VMM10	------------	n/a
VMM12	Tyrosinase	A1
VMM14	Tyrosinase	unknown
VMM15	Tyrosinase	A1
	MART-1	unknown
VMM18	Pmel17	A3
	MART-1	A3
VMM19	------------	n/a
VMM21	MART-1	unknown
VMM30	Tyrosinase	unknown
VMM39	------------	n/a
DM 331	------------	n/a

EXAMPLE A1-2

CTL from HLA-A1+ human melanoma patients recognize tyrosinase-derived epitopes

Melanoma-reactive CTL lines were generated from human
5   melanoma patients and tested for reactivity against
autologous and allogeneic melanomas and autologous and
allogeneic B-LCL following transient expression of the
melanocyte differentiation proteins Pmel-17/gp100, MART-
1/Melan-A, and tyrosinase. These CTL lines were tested as
10 soon as residual NK and lymphokine activated killer
activities were low and reactivity against autologous
melanoma could be demonstrated, typically at 25 to 40 days in
culture. As shown in Table 201, reactivity against
tyrosinase-derived epitopes was observed for five of the six
15 HLA-A1+ CTL lines. The one HLA-A1+ CTL line that did not
demonstrate reactivity against tyrosinase was derived from a
patient whose tumor line, DM331, does not express tyrosinase
or any other defined melanocyte differentiation proteins.
Thus, tyrosinase-derived epitopes were recognized by CTL from
20 all HLA-A1-positive patients whose tumors express tyrosinase.
CTL lines from melanoma patients recognize tyrosinase encoded peptides restricted by HLA-A1

Of the HLA-A1+ CTL lines recognizing tyrosinase epitopes,
VMM12 CTL and VMM15 CTL lines were sufficiently available to
25 permit detailed analysis. To determine which of the class I
MHC alleles was responsible for presentation of tyrosinase
epitopes to these two CTL lines, the tyrosinase-vaccinia
constructs were evaluated in C1R cells transfected with one
of the class I MHC alleles expressed on the patient's tumor.
30 Following infection with the recombinant vaccinia virus
expressing full-length tyrosinase, the HLA-A1 transfectant of
C1R was recognized and lysed by VMM12 CTL and VMM15 CTL, but
HLA-A3, -B7, and -B8 C1R transfectants were not recognized
under identical conditions. Thus, HLA-A1 is a restriction
35 element for the recognition of tyrosinase by CTL lines VMM12
and VMM15.

VMM15 CTL were also evaluated for recognition of a panel
of target cells. Lysis depended on the expression of HLA-A1
and the expression of tyrosinase. In particular, there is no

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lysis of the tyrosinase-negative melanoma Na8Mel, but there
was significant lysis of the tyrosinase-transfected cell line
Na8Mel+Tyr. Both Na8Mel and Na8Mel+Tyr express HLA-A1. The
melanoma line DM331, which is HLA-A1+ and tyrosinase

5 negative, is not lysed, whereas the HLA-A1+/tyrosinase+
melanomas HT144 and VMM14 are lysed. VMM12 tumor cells
variably lose expression of HLA-A1, and are not lysed in this
assay but have been lysed by VMM15 CTL in other assays when
HLA-A1 is expressed. DM6 is a tyrosinase+/HLA-A1-negative

10 melanoma and is not lysed. In several other assays, SkMel24,
another tyrosinase-negative, HLA-A1+ melanoma, was not lysed
by this CTL line. Thus, tyrosinase expression was both
necessary and sufficient for lysis of HLA-A1+ target cells by
VMM15 CTL.

15 *HLA-A1-restricted CTL recognize peptides containing the
sequence KCDICTDEY*

      To identify the HLA-A1-restricted peptide epitopes
recognized by VMM12 and VMM15 CTL, we scanned the tyrosinase
protein sequence for peptides matching published HLA-A1

20 binding motifs . For nine-residue peptides, these motifs can
be summarized as follows: threonine, serine, or methionine at
position two; aspartate, glutamate, alanine, or serine at
position three; and tyrosine at the carboxyl terminus. Also
included in this evaluation were peptides containing more

25 than 9 residues. Since the C-terminal tyrosine residue
appears to be invariant for HLA-A1 binding peptides, we
predicted that longer peptides may retain the C-terminal
tyrosine but would be extended from the N-terminus. In a
previous study of HLA-A2 restricted epitopes, a post-

30 translational modification of tyrosinase involving a
deamination reaction resulted in the conversion of an
asparagine residue into aspartate. Therefore, in addition to
testing the wild-type version of potential HLA-A1 binding
peptides that contained N-linked glycosylation sites (..N-x-

35 S/T..), we also tested variants of these peptides with
aspartate (D) substituted for asparagine (N). With these
considerations, over 100 peptides matching this motif and
ranging in length from eight to twelve amino acids were

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synthesized and tested for their ability to reconstitute the
epitope recognized by VMM15 CTL. None of the first set of
peptides tested had activity; so additional peptides
synthesized included a number that fit the binding motif
5   loosely: containing only threonine or serine at position 2,
only an acidic residue at position 3, or only the C-terminal
tyrosine. In one experiment, three of 48 peptides tested
were recognized: KCDICTDEY, DAEKCDICTDEY, and EKCDICTDEY, all
of which contained the core nonamer sequence KCDICTDEY
10   (Figure 5). These three peptides containing the KCDICTDEY
sequence will, collectively, be referred to as KCDICTDEY-
related peptides.
        These three synthetic peptides were purified by HPLC,
and evaluated in a dose-titration assay (Figure 8).
15   Recognition of C1R-A1 cells pulsed with each of these
peptides was indistinguishable by VMM15 CTL and, at higher
peptide concentrations, targets pulsed with each of these
three peptides were recognized at levels comparable to the
autologous tumor cells (Figure 8). Similarly, we found that
20   lysis of target cells pulsed with DAEKCDJCTDEY (50 μg/ml) can
be inhibited by competition with the wild-type VMM15 tumor
cells in a cold target inhibition assay. Thus, the epitope
reconstituted by a KCDICTDEY-related peptide corresponds to
the epitope presented naturally on VMM15 tumor cells.
25   However, the concentration of peptide required for
reconstitution was high, with half-maximal lysis observed at
2μg/ml (approximately 2 μM). These peptides are
distinguished from most other CTL epitopes by containing two
cysteine residues. Since most other defined epitopes for
30   melanoma-reactive CTL can be reconstituted at nanomolar
concentrations or lower this raised a question of whether the
KCDICTDEY-containing peptides actually constituted the true
epitope for these CTL, and whether they were naturally
processed and presented in association with HLA-A1.

35   _Identification of DAEKCDICTDEY as the naturally presented_
     epitope in association with HLA-A1 on melanoma cells. To
     determine whether any of the KCDICTDEY-containing peptides

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are actually processed and presented by melanoma cells in association with HLA-A1 molecules, HLA-A1-associated peptides were isolated, reduced in DTT to remove possible modifications on the sulfhydryl groups of cysteine residues,

5   then fractionated by HPLC. Synthetic forms of the three KCDICTDEY-containing peptides were also fractionated under identical conditions to identify their elution point relative to the naturally-processed peptides in the tumor cell extracts.   Fractions 17-20 were identified as representing

10 the elution points for all of these KCDICTDEY-containing peptides. The nonamer and decamer were found in fractions 17 and 18; the undecamer was found in fraction 18, the dodecamer was found in fractions 19 and 20.   Thus, aliquots of those fractions were evaluated by tandem mass spectrometry to

15 determine whether any of the KCDICTDEY peptides were present in the extracts.   CAD spectra were generated for species with m/z ratios 545, 610, 645, and 702, corresponding to the nonamer (KCDICTDEY), decamer (EKCDICTDEY), undecamer (AEKCDICTDEY), and dodecamer peptides (DAEKCDICTDEY),

20 respectively, in each of these fractions. An ion with m/z 702 was identified in fraction 20, and the CAD spectrum of that species confirmed its identity as DAEKCDICTDEY, the dodecamer peptide recognized by VMM15 CTL.   In addition, the decamer peptide EKCDICTDEY was also identified, but at a lower copy

25 number, in this extract.   Neither the nonamer peptide KCDICTDEY nor the undecamer peptide AEKCDICTDEY was found.

These KCDICTDEY-containing peptides include a high number of acidic residues (two aspartic acids (D) and one glutamic acid (E)) and two cysteine (C) residues.   When the

30 DAEKCDICTEY peptide was identified by mass spectrometry, it contained a disulfide bond between the two cysteine residues, which could be reduced by treatment with dithiothreitol (DTT) such that the linear configuration with two free cysteine residues was returned.   The internal disulfide bond forms a

35 cyclized central portion of the molecule and results in a loss of two hydrogen atoms, and, therefore, ions containing both cysteines are two mass units smaller than in the reduced form.   In addition, in the cyclized peptide, both cysteine

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residues remain together after collision activated
dissociation; thus, the CAD spectrum for this species lacks
the b_5 (DAEKC+), b_6(DAEKCD+), and b_7(DAEKCDI+) ions as well
as the y_5(+CTDEY), y_6(+ICTDEY), and y_7(+DICTDEY) ions. On the
5   other hand, the CAD spectrum for the reduced species contains
detectable b_5, b_6, b_7, and y_5 ions.   The CAD spectrum for the
reduced species is identical to the CAD spectrum for the
synthetic DAEKCDICTDEY peptide.

<u>Substitution of serine or alanine for the more N-terminal</u>
10 <u>cysteine residue markedly enhances reconstitution of the CTL</u>
<u>epitope</u>
        By direct evaluation of peptides eluted from HLA-A1
molecules, it was not possible to determine definitively
which of the KCDICTDEY-related peptides was naturally
15 processed and presented in association with HLA-A1,
suggesting that they are present in low quantity or have been
modified.   Subsequent studies thus addressed potential
modifications of both the nonamer KCDICTDEY and the dodecamer
DAEKCDICTDEY.   Spontaneous modification of free cysteine
20 residues by disulfide bond formation will occur in medium
containing cystine (C-C) or glutathione, such as RPMI or
human serum.   Thus, we examined the effect of substituting
serine or alanine for one or both cysteine residues in the
synthetic KCDICTDEY-related peptides in an effort to prevent
25 the formation of disulfide bonds.
        The synthetic nonamer with the wild-type sequence
(KCDICTDEY) induced half-maximal lysis at 2 to 20μM.
However, when the cysteine closer to the N-terminus (position
2) was replaced with serine or alanine (KSDICTDEY or
30 KADICTDEY), half-maximal lysis was observed after incubation
of targets with 100- to 1000-fold lower concentrations (2 and
20nM, respectively; Fig. 8A). On the other hand, peptides
with alanine or serine substituted for the cysteine distal to
the N-terminus, at position (KCDISTDEY or KCDIATDEY) were not
35 discernibly more effective than the wild-type peptide.
Nonamer peptides with alanine or serine substituted for both
cysteine residues were actually less effective than the wild-

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type peptide.

Similarly, the dodecamer with the wild-type sequence
(DAEKCDICTDEY) required 10μM for half-maximal sensitization
of target cells for lysis. However, when the cysteine closer
5   to the N terminus (position 5) was replaced with alanine
(DAEKADICTDEY), half-maximal lysis was observed after
incubation of targets with approximately 100 nM (Fig. 5B).
On the other hand, alanine substitution for the cysteine
closer to the C-terminus, at position 8 (DAEKCDIATDEY),
10 prevented recognition at every concentration tested. These
data demonstrate that the more C-terminal cysteine (residue
247 of tyrosinase) is critical to the formation of the CTL
epitope, raising the possibility that spontaneous
modifications of that residue by disulfide bond formation may
15 be integral to the actual epitope.

Because of the similar effects on CTL recognition
observed with amino acid substitutions for the more N-
terminal cysteine residue in both the dodecamer DAEKCDICTDEY
and the nonamer KCDICTDEY, and because of the similar dose-
20 titration curves observed for CTL recognition (Fig. 8), we
hypothesized that residues 243 to 251 (KCDICTDEY) of both
peptides adopt a similar conformation when bound to HLA-A1.
A cold target inhibition assay was performed with peptide-
pulsed target cells to test that hypothesis. As shown in
25 Figure 9, unlabeled target cells pulsed with the dodecamer
effectively inhibited lysis of Cr-labeled targets pulsed with
the nonamer, at a level equivalent to the inhibition induced
by cold targets pulsed with the nonamer itself. Recognition
of labeled targets pulsed with the dodecamer DAEKSDICTDEY was
30 similarly inhibited by unlabeled targets pulsed with the
nonamer KSDICTDEY. These data suggest that the conformation
of the peptide-MHC complex within the region recognized by
the TCR of VMM15 is, therefore, similar or identical for the
nonamer and dodecamer peptides.

35 **Discussion**

We have shown previously that the melanocyte
differentiation protein Pmel-17/gp100 is a source of epitopes

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for melanoma-reactive CTL restricted by HLA-A*0201 and HLA-A3. Using vaccinia constructs encoding this protein and CTL lines from patients with varied MHC profiles, we identified the Pmel-17/gp100 peptide ALLAVGATK as an epitope for A3-
5   restricted CTL. We now provide evidence that tyrosinase-derived peptides are frequently recognized by HLA-A1* CTL. Among HLA-A1* patients, CTL recognition of tyrosinase epitopes was evident in all cases, except when the autologous tumor itself failed to express tyrosinase. By screening over 100
10 synthetic peptides derived from the tyrosinase sequence, we found that the peptides KCDICTDEY, EDCDICTDEY, and DAEKCDICTDEY were all recognized by A1-restricted melanoma-reactive CTL.

The naturally processed epitope may be a nonamer or may
15 be as long as a dodecamer. Peptides containing 12 residues are uncommon among described class I MHC-associated peptides, and we are unaware of previously reported examples of dodecamer peptides representing CTL epitopes. Dodecamer peptides have been identified in association with cells
20 containing Ag-processing defects, where a TAP-independent pathway has been implicated in their processing and presentation. Also, in cells with normal Ag-processing machinery, dodecamers associated with HLA-A1 and HLA-A11 have been described. A dodecamer from cytochrome C oxidase that
25 is associated with HLA-A1 contains putative anchor residues at positions 3 and 12 (YTDYGGLIFNSY), see Engelhard, et al., Currl. Opin. Immunol., 6:13 (1994). Thus, that peptide is predicted to bind to the HLA-A1 molecule with a kink between residues 3 and 12 to allow for the extra length. The
30 synthetic DAEKCDICTDEY peptide, however, could potentially bind to HLA-A1 in either of two conformations. While the tyrosine residue at the C terminus is almost certain to function as the C-terminal anchor residue, the motif requirement for an acid residue at position 3 could be
35 satisfied either by the glutamic acid (E) at position 3 or by the aspartic acid (D) at position 6. In the former case, the central portion of the peptide would have to be kinked and would extend out of the plane of the binding groove, a

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conformation that could b stabilized or induced by a
disulfide bond between the two cysteine residues. If,
however, the aspartic acid residue at position 6 functions as
an anchor residue, the three N-terminal residues (DAE) would
5   be expected to extend up and out of the binding groove, while
the rest of the peptide interacts in the groove in a
conformation resembling that of the nonamer peptide.

        If the dominant conformation of the dodecamer is kinked,
then it would be anticipated that CTL recognition would be
10 sensitive to the peptide structure between residues 5 and 12,
and that CTL recognizing that peptide would not cross-react
with target cells pulsed with the synthetic nonamer lacking
the N-terminal DAE residues. However, CTL reactive against
the dodecamer were equally reactive against both the decamer
15 EKCDICTDEY and the nonamer KCDICTDEY. While these studies
were performed with a CTL line, which probably contained
distinct CTL subpopulations, our cold target inhibition
assays provide evidence that both the nonamer and dodecamer
peptides are recognized by the same CTL. We have also
20 observed that dodecamers containing glycine substituted for
alanine or glutamate at positions 2 and 3 were recognized at
levels comparable to those of the wild-type peptide (data not
shown). Therefore, the nonamer and dodecamer peptides
probably exist in similar conformation when bound to HLA-A1,
25 such that the three amino-terminal residues of the dodecamer
would extend out of the binding groove, and residues 4
through 12 would be expected to lie flat, in a manner
analogous to the configuration expected for the nonamer
peptide.

30   Regardless of the precise length and conformation of the
peptides bound to the MHC, the KCDICTDEY-related peptides are
unusual in containing two cysteine residues. In general,
sulfhydryl groups on cysteine residues are susceptible to
derivatization with sulfhydryl groups on other biologic
35 molecules such as cystine or glutathione, both of which are
present in human serum and RPMI. Especially, since formation
of disulfide bonds is favored at mildly basic pH and in the
presence of oxygen, both the in vivo setting (pH 7.4, pO_2 40-

100 mm Hg) and the standard culture conditions in vitro would
favor modification of cysteine residues with exposed side
chains. To assess the roles of the two cysteine residues in
the KCDICTDEY-related peptides with respect to T cell

5   recognition, a series of experiments was performed comparing
the naturally occurring peptides with peptides containing
amino acid substitutions. Substitution of serine or alanine
for the more N-terminal cysteine prevents potential
modifications of that residue, including formation of an

10 internal disulfide bond. After making such a substitution,
both the dodecamer and the nonamer were capable of
reconstituting CTL epitopes at concentrations 2 to 3 logs
lower than the unsubstituted peptides. Thus, we hypothesize
that disulfide bonds involving this more N-terminal cysteine

15 residue negatively affect binding of exogenous peptide to the
HLA-A1 molecule, and that in the naturally processed peptide,
the cysteine residue at that position (position 2 of
KCDICTDEY, residue 244 of tyrosinase) is not modified,
perhaps being protected within the binding groove of HLA-A1.

20 Similarly, the more C-terminal cysteine would be anticipated
to point upward and to have an exposed side chain. In
cystine- and glutathione-rich medium, such as RPMI (in vitro)
or human serum (in vivo), the more C-terminal cysteine may
well be modified by cysteinylation or by derivatization with

25 another molecule containing a sulfhydryl group. T cell
recognition of that epitope may depend on such a
modification. Indeed, as shown in Figure 8, recognition by
VMM15 CTL is acutely sensitive to the presence of a cysteine
residue at this position (KSDICTDEY vs KSDISTDEY).

30   Peptides load onto NHC molecules in the oxidative
environment of the endoplasmic reticulum (ER), where
modification of cysteine residues by reversible formation of
disulfide bonds would be expected. However, at least one
enzyme in the ER, protein disulfide isomerase, can catalyze

35 dissociation of disulfide bonds.

The KCDICTDEY consensus sequence is unusual in the large
number of polar amino acid residues, including two aspartic
acid residues, one glutamic acid residue, and two cysteine

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residues. The tyrosine residue at the C-terminus (position 9 of KCDICTDEY), and the aspartic acid at position 3 of the KCDICTDEY peptide are critical residues for binding to the MHC; so we conclude that the A1 molecule binds KCDICTDEY in
5   its binding groove and that when the dodecamer peptide DAEKCDICTDEY binds to HLA-A1, the N-terminal residues DAE extend out of the pocket. Alternatively, one could postulate that this peptide could bind to HLA-A1 in the cyclized conformation caused by disulfide linkage of the 2 cysteine
10   residues. The glutamic acid (E) residue at position 3 and the tyrosine at position 12 could fit the binding motif if enough of a kink were created in the peptide by the disulfide bond. However, CTL reactive against this epitope were equally reactive against the nonamer KCDICTDEY (Figure 7),
15   suggesting that the configuration of the epitope is the same for these two peptides, and that the epitope is independent of the excess residues extending out of the A1-binding groove. This is further supported by the finding that substitution of serine or alanine for the cysteine at residue
20   244, which would prevent the internal disulfide bond, dramatically increased the ability of this peptide to reconstitute the CTL epitope. Thus, the CTL appear to have been sensitized against an epitope in which the peptide has not formed a disulfide bond. Further, since the nonamer and
25   the dodecamer are equally well recognized by CTL, the epitope appears to be independent of the N-terminal 3 amino acids (DAE).

Once peptides are dissociated from the MHC, especially in the presence of oxygen or in slightly basic conditions
30   (eg., exposed to air, or in vivo conditions: pH 7.4), modifications of cysteine residues by formation of disulfide bonds may be anticipated. At a molecular level, proximity likely favors formation of the internal disulfide bonds, resulting in cyclization of these KCDICTDEY peptides. It is
35   also possible that either of the cysteine residues in this peptide may be modified in the endoplasmic reticulum, such as by addition of an acyl group, or that modification of exposed sulfhydryl groups may occur while the peptide:MHC complex is

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exposed to serum components in vivo, where cyclization would be inhibited by association with the MHC. The more C-terminal cysteine would be easily accessible to derivatization by glutathione or cystine. Such modifications

5   were not directly identified by mass spectrometry, but it remains a possibility that the optimal epitope in vivo includes a spontaneous modification of the more C-terminal cysteine residue.

We predict that those peptides not containing internal

10   disulfide bonds or other modifications of the more N-terminal cysteine are those capable of binding to the nascent MHC molecules for presentation at the cell surface. Whether there are specific molecular chaperones favoring this process in the ER remains to be demonstrated.

15   Aside from what happens during normal Ag processing and presentation, synthetic peptides free in solution are susceptible to modifications of cysteine residues by formation of disulfide bonds, especially in the presence of oxygen or in slightly basic conditions (e.g., exposed to are

20   or in vivo). Because of the proximity of the two cysteine residues, the likelihood of interaction between them probably exceeds the likelihood of interaction with another molecular species in solution. In fact, intramolecular disulfide bonds between cysteine residues in short peptides containing the

25   sequence - CXYC- are highly favored at concentrations up to 200 μM in neutral pH solutions and are facilitated by air oxidation of the cysteine residues. The resulting cyclized form of KCDICTDEY-related peptides may not be capable of binding to the HLA-A1 molecule or, if bound, would probably

30   result in a molecular conformation different from that of the epitope recognized by CTL. The fact that cyclization and other cysteine modifications occur readily may well explain the high concentration of KCDICTDEY-related peptides required for sensitization of CIR-A1 target cells in vitro and would

35   imply similar results in vivo if a tumor vaccine using the wild-type peptide were administered.

On the other hand, peptides containing substitutions of serine or alanine for the more N-terminal cysteine induced

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half-maximal lysis of target cells at 1 to 20 nM, which
compares favorably to peptide concentrations required for
reconstitution of the MAGE-1 epitope EADPTGHSY on HLA-A1
(half maximal lysis at ~ 10 nM) and the MART-1 peptide
5   AAGIGILTV on HLA-A*0201 (half-maximal lysis at ~1-100nM). We
believe that replacement of the more N-terminal cysteine
probably enables purified peptides to bind to cell surface
MHC molecules in a manner that more accurately mimics the
configuration of peptides binding to nascent MHC molecules in
10   the ER.
        A concern with these KCDICTDEY-containing peptides, in
terms of their potential usefulness in vaccine therapy, is
that high concentrations were required for sensitization of
target cells, with half-maximal lysis requiring approximately
15   2 uM concentrations. As described above, the two cysteine
residues in these peptides likely explain some of their
unusual features. The more C-terminal cysteine appears to be
susceptible to interaction in vivo with sulfhydryl groups on
biologic molecules such as cystine (present in human serum at
20   276 uM) or glutathione, and we have shown that the free
peptide is capable of forming an intramolecular disulfide
bond, resulting in cyclization of the peptide. Because of
the proximity of these two cysteine residues, the likelihood
of interaction between them probably exceeds the likelihood
25   of interaction between one of them and another molecular
species in solution. Intramolecular disulfide bonds between
cysteine residues in short peptides containing the sequence -
CxyC- are highly favored at concentrations at and below 200uM
in neutral pH solutions, and are facilitated by air oxidation
30   of the cysteine residues. This cyclic form is not likely to
be capable of binding to the HLA-A1 molecule, and its
formation likely explains the high concentration required for
sensitization of C1R-A1 target cells in vitro. Although the
wild-type forms of these peptides require high concentrations
35   for reconstitution, the substituted forms of these peptides
induce half-maximal lysis of target cells at 1-20 nM
concentrations, which compares favorably to reconstitution
of the MAGE-1 epitope EADPTGHSY on HLA-A1, (half-maximal at

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approximately 10 nM) and the MART-1 peptide AAGIGILTV on
HLA-A2 (at approximately 1-100 nM).

The significantly greater recognition of KADICTDEY,
KSDICTDEY, and DAEKADICTDEY compared to KCDICTDEY and

5   DAEKCDICTDEY (Figures 8 and 9), supports the hypothesis that
modifications of the more N-terminal cysteine residue
interferes substantially with reconstitution of the epitope.
In vivo, the naturally processed peptide would bind to the A1
molecule in the endoplasmic reticulum, and the more N-

10 terminal cysteine would be protected from modification by
being buried in the peptide-binding groove. Thus,
substituting alanine or serine for cysteine in this position,
when working with the free peptide, may protect that residue
from modifications until the peptide can bind to the A1

15 molecule.

On the other hand, the more C-terminal cysteine is
anticipated to point upwards towards the T cell receptor.
This residue would be susceptible to modification at the cell
surface while bound to the MHC molecule. In cystine-

20 containing media, such as RPMI (in vitro) or human serum (in
vivo), the more C-terminal cysteine may well be modified by
cysteinylation or by derivitization with another molecule
containing a sulfhydryl group. T-cell recognition of that
epitope may depend partly on that modification, but may not

25 require that modification.

In summary, three peptides containing the KCDICTDEY
sequence are capable of reconstituting an epitope for HLA-A1-
restricted melanoma-reactive CTL, but only the decamer
EKCDICTDEY and the dodecamer DAEKCDICTDEY appear to be

30 naturally processed and presented. Modifications of the more
C-terminal cysteine residue by cysteinylation is likely to
occur in vitro and in vivo, and this modification may well
affect T-cell recognition. Modifications of the more N-
terminal cysteine residue, however, are not believed to occur

35 in vivo during normal processing and presentation but may
occur when the peptide is free in solution. Modifications,
such as intramolecular disulfide bonds leading to cyclization
of the peptide, probably inhibit markedly the binding of that

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peptide to HLA-A1 at the cell surface. These observations
have several implications. First, synthetic peptides that
contain one or more cysteine residues may not be capable of
reconstituting epitopes for CTL under standard conditions and

5   may, therefore, be difficult to identify as CTL epitopes
using standard methods unless the described modifications are
anticipated. Second, preventing disulfide bond formation by
modifying cysteine-containing peptides will improve their
usefulness. For this particular peptide, it may be useful to

10   immunize with the nonamer peptide containing serine or
alanine at position 2, in place of the first cysteine.

As demonstrated by our data with the wild-type and
substituted forms of KCDICTDEY-related peptides, cysteine
residues can both positively and negatively affect MHC

15   binding and/or T cell recognition. This suggests that
potential problems with using cysteine-containing peptides as
immunogens may be overcome by modifications of those cysteine
residues. In particular, immunization with a peptide
containing serine or alanine in place only of a non-essential

20   cysteine may result in more effective loading onto MHC
molecules of APC.

Table 201

ANTIGEN REACTIVITY OBSERVED[a]

CTL Line	MHC typing of CTL donor	MCH class I molecules evaluated	Pmel-17/ gp100	MART-1/Melan-A	tyrosinase
DM331	-A1,2;-B15,B62	-A1,2	-	-	-
VMM12	-A1,A3;-B7,B14	*	-	-	+
VMM14	-A1,25;-B8,B48	-A1,25;-B8	-	-	+
VMM15	-A1,25;-B8,B18	*	-	+	+
VMM18	-A3,31/33;-B60;-C3	*	+	+	-
VMM19	-A24;-B35,55;-C3	*	-	-	-
VMM21	-A1,2;-B7,37	*	-	+	+
VMM34	-A3,30;-B21,70	-A3	-	-	-
VMM39	-A2,3;-B7	-A2,3;-B7	-	-	-
VMM40	-A1,2;-B27,57;-C37	*	-	-	+

Positive values indicate that target cells expressing the antigen listed, via a vaccinia construct, were lysed (at an E:T ratio of 30:1) at a level at least 10% higher than background lysis of the target cell infected with an irrelevant vaccinia construct.

* indicates all MHC were evaluated: these CTL were evaluated for recognition of autologous EBV-B cell lines infected with each of the vaccinia constructs listed.

REMARKS

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other

5 *references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the contents of the references cited within the references cited herein are also entirely incorporated by reference.*

10 *Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.*

15 *The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such*

20 *specific embodiments, without undue experimentation, without departing from the generic concept of the present invention. Therefore, such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and*

25 *guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings*

30 *and guidance presented herein.*

For immunological techniques generally, see Coligan, et al, <u>Current Protocols in Immunology</u> (NIH: 994); Harlow and Lane, <u>Antibodies:A laboratory Manual</u> (Cold Spring Harbor Lab.: 1988).

35 *An immunogen is deemed not to occur in nature, even though its component epitopes do occur in nature, if the immunogen itself, as a single molecule, does not occur in nature. For example, a conjugate of KCDICTDEY to albumin does not occur in nature even though KCDICTDEY is a fragment of tyrosinase*

which is generated by the immune system processing of
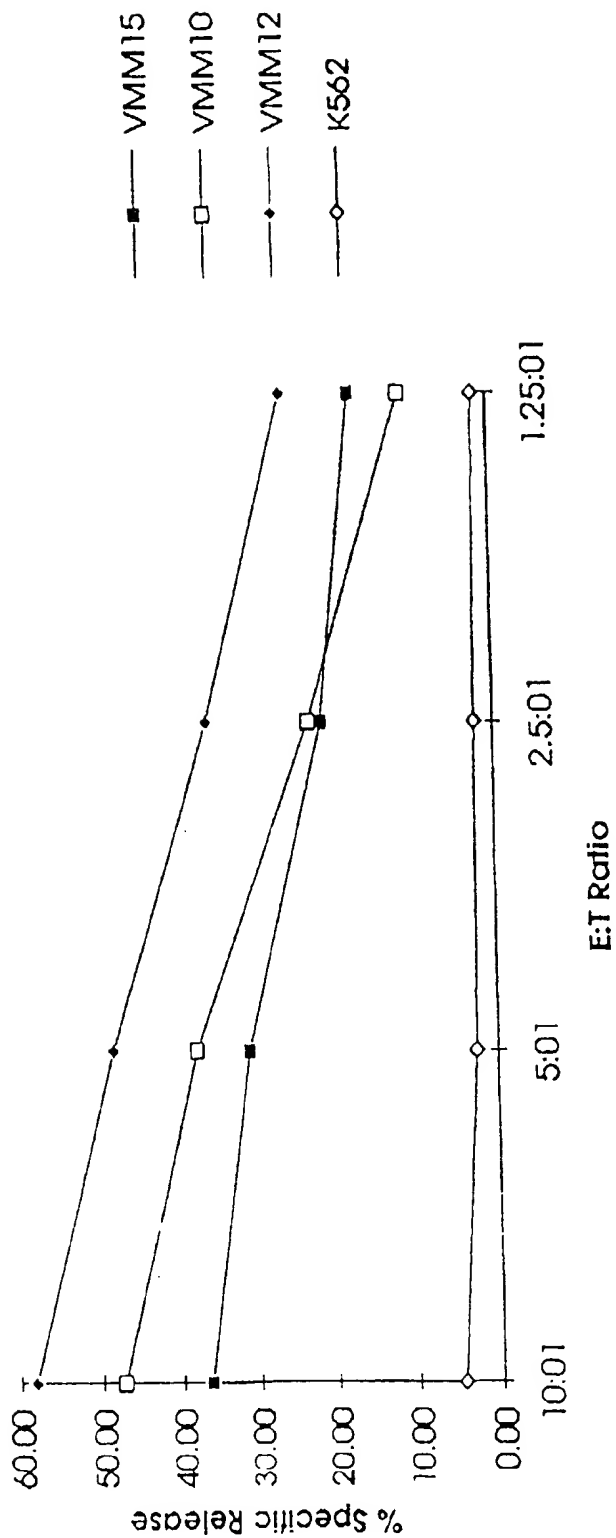*tyrosinase and complexes with MHC.*

What is claimed is:

1. A disease-specific immunogen which comprises a disease-specific CTL epitope which is a cysteine-depleted mutant of a native disease-specific CTL epitope, and said immunogen
5   itself does not occur in nature.

2. The immunogen of claim 1 wherein said native epitope is a cysteine-containing tyrosinase epitope.

3. The immunogen of claim 1 wherein at least one of said cysteine-depleted epitopes is an A1-restricted epitope.

10 4. The immunogen of claim 1 which is tumor-specific.

5. The immunogen of claim 1 which is melanoma-specific.

6. The immunogen of claim 1 wherein one of said native epitopes is (DAE) KCDICTDEY.

7. The immunogen of claim 1 wherein at least one cysteine of
15 the native epitope is replaced by Ala, Thr, Ser, Gly or Met.

8. An expression vector which comprises a gene encoding a disease-specific immunogen according to any of claims 1-7, operably linked to one or more expression control sequences, whereby said gene may be expressed in a mammalian subject, in
20 which subject said expressed immunogen is capable of eliciting a melanoma-specific CTL response.

9. The immunogen of claim 1, which is an isolated, purified, or synthetic peptide of about 9 to about 15 residues in length.

25 10. A composition comprising a peptide according to claim 9 and a class I MHC molecule, whereby T lymphocytes may be stimulated by said peptide.

11. T lymphocytes stimulated by an immunogen according to claim 1.

30 12. A method of protecting a mammal against a disease which comprises administering to a mammal in need thereof a therapeutically or prophylactically effective amount of one or more of:

(a) The immunogen of claim 1,

35 (b) an expression vector which comprises a gene encoding a suitable disease-specific immunogen according to claim 1, operably linked to one or more expression control sequences, whereby said gene may be expressed in a mammalian subject, in which subject said expressed immunogen is capable of
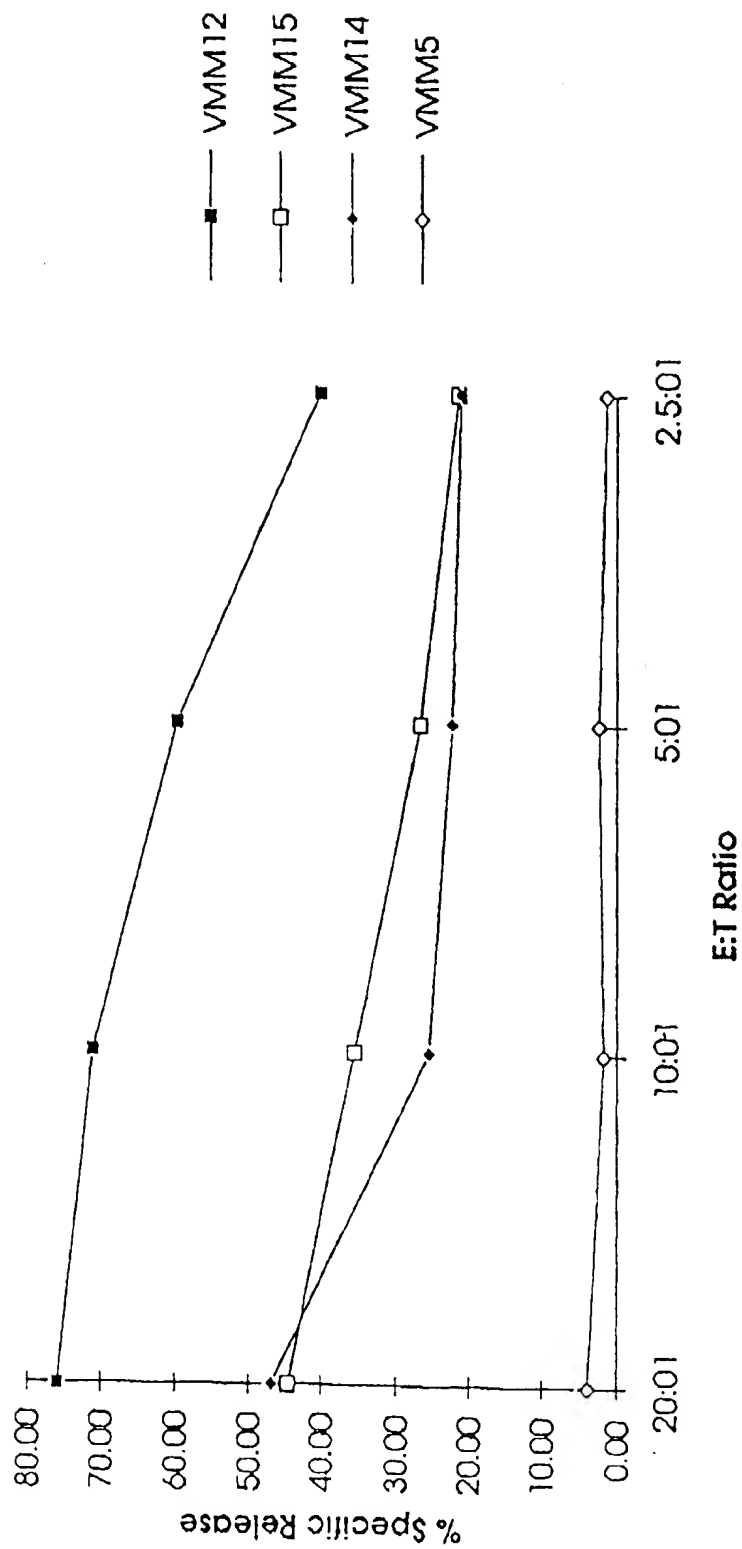
eliciting a disease-specific CTL response, or

(c) T lymphocytes stimulated by an immunogen according to claim 1.

VMM12 1.71 d36 (Assay34 14/7/93)

VMM15 2.3 d46 (Assay67 13.10.93)
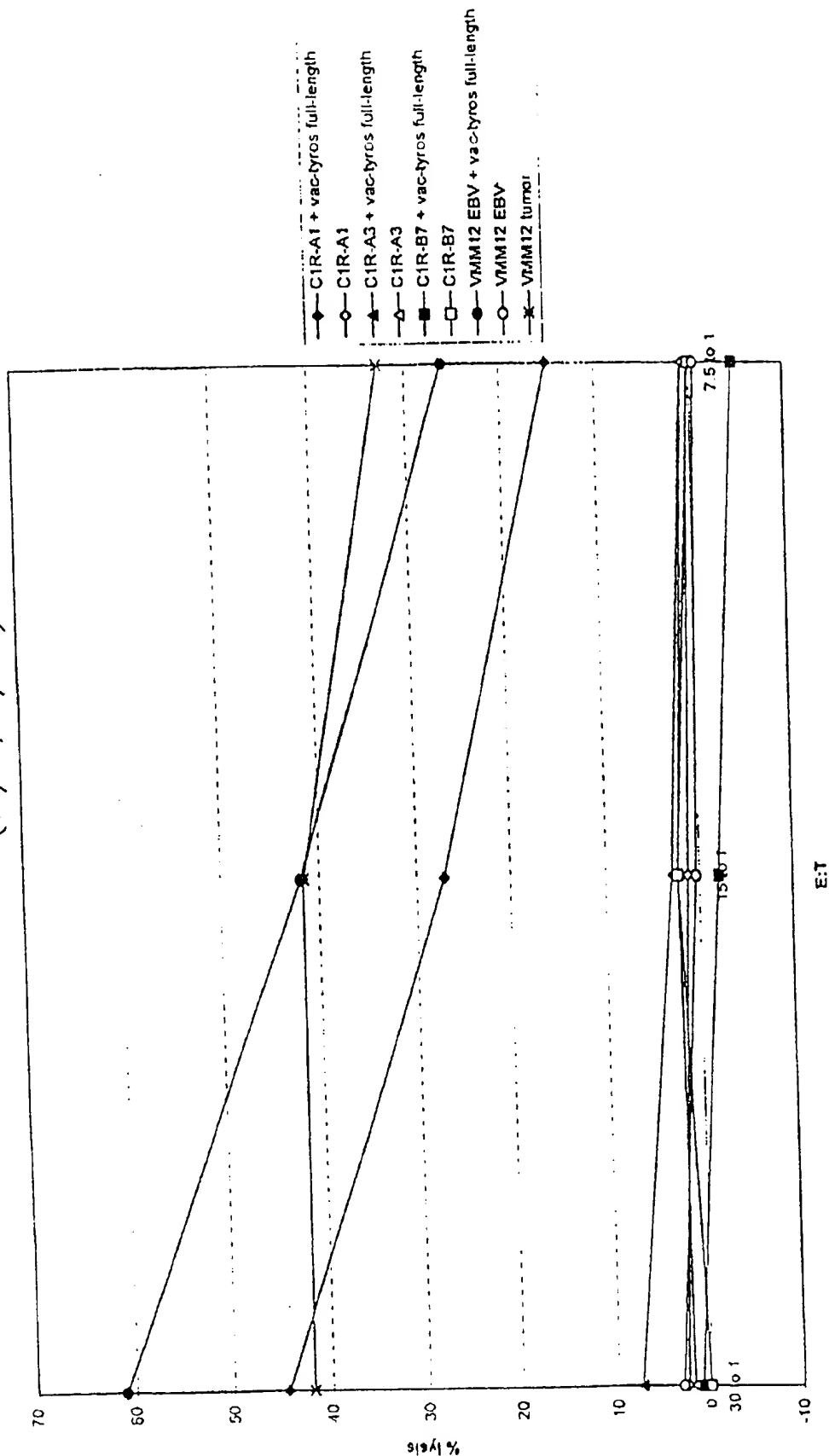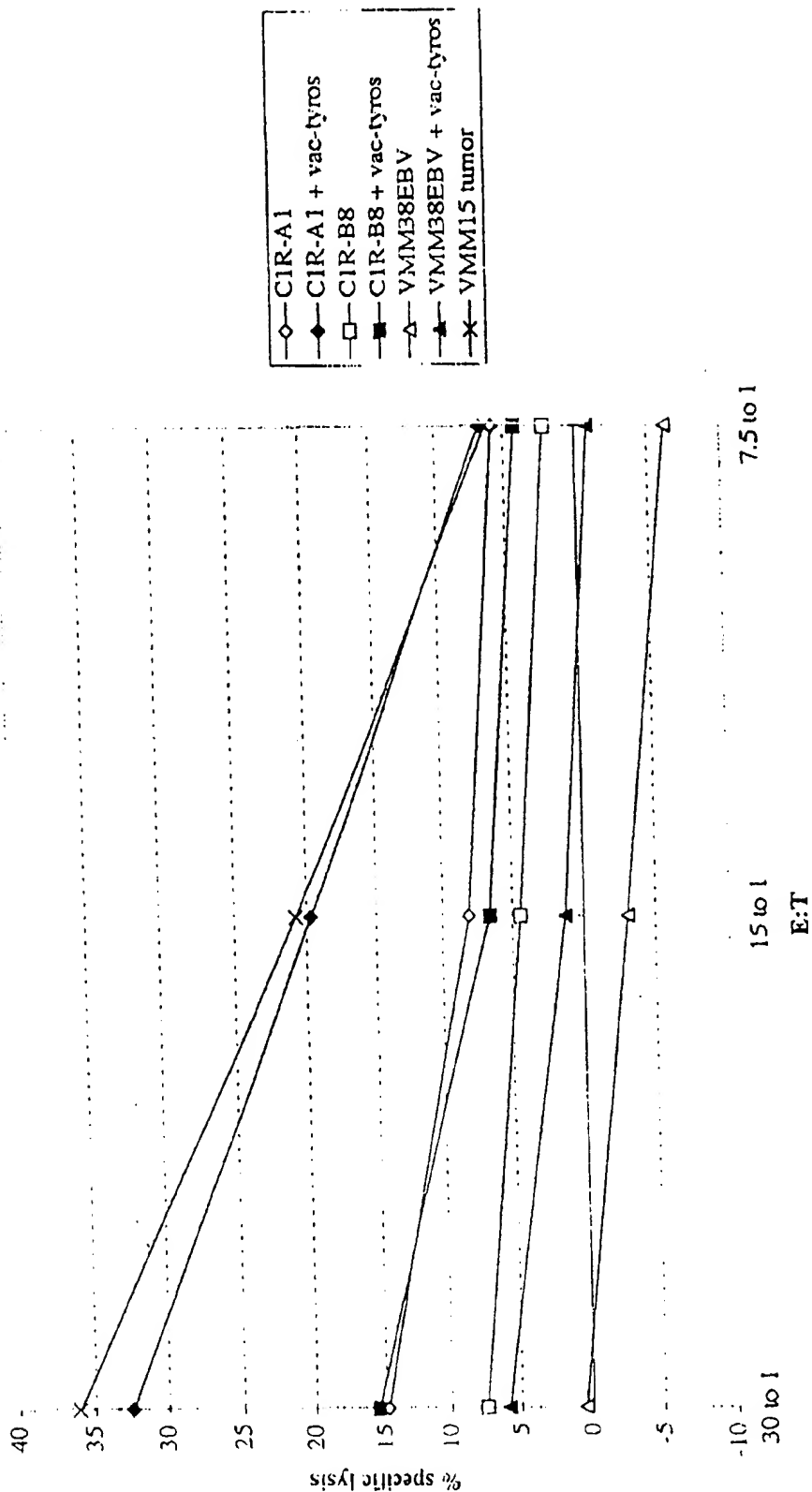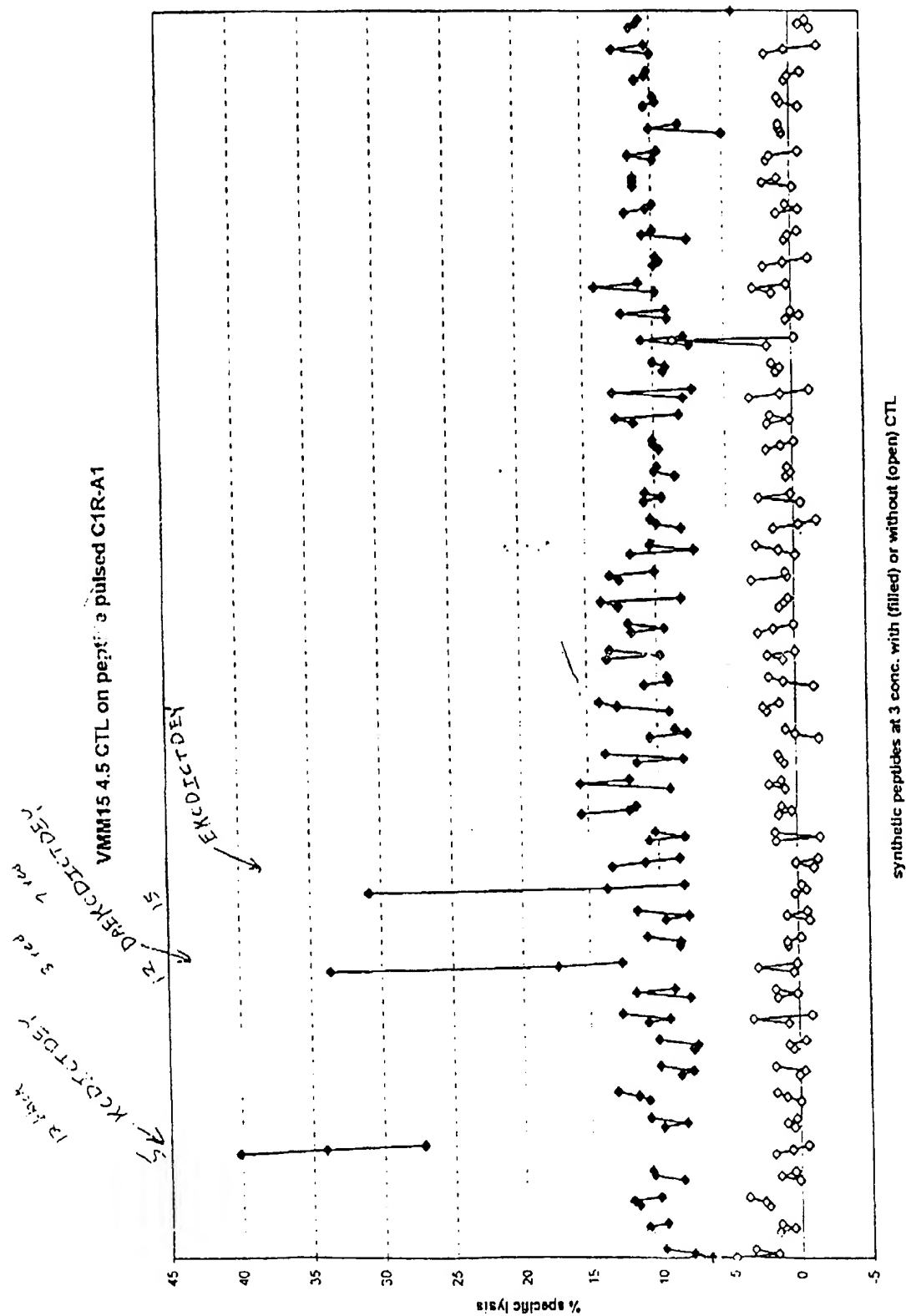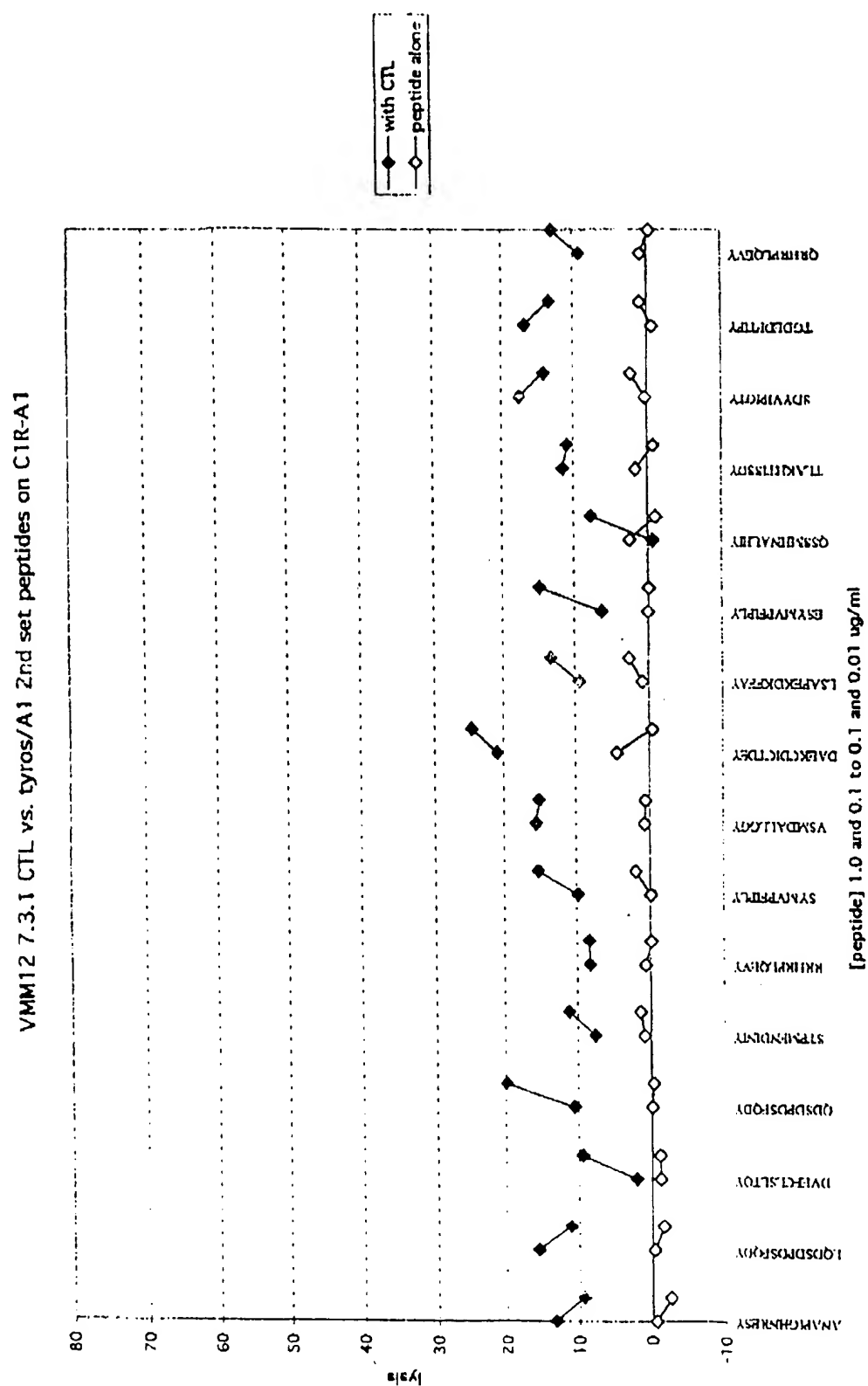
VMM12 1.71 CTL (d80)

(A1, A3, B7, B14)

E:T

Page 1

Figure 2B

VMM15 CTL recog. of tyrosinase

Legend:
- ◇ C1R-A1
- ◆ C1R-A1 + vac-tyros
- □ C1R-B8
- ■ C1R-B8 + vac-tyros
- △ VMM38EBV
- ▲ VMM38EBV + vac-tyros
- ✕ VMM15 tumor

% specific lysis

E:T

Page 1

VMM15 4.5 CTL on peptide pulsed C1R-A1

synthetic peptides at 3 conc. with (filled) or without (open) CTL

Page 1

VMM12 7.3.1 CTL vs. tyros/A1 2nd set peptides on C1R-A1

[peptide] 1.0 and 0.1 to 0.1 and 0.01 ug/ml

Page 1

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  1    MLLAVLYCLL  WSFQTSAGHF  PRACVSSKNL  MEKECCPPWS  GDRSPCGQLS
 51    GRGSCQNILL  SNAPLGPQFP  FTGVDDRESW  PSVFYNRTCQ  CSGNFMGFNC
101    GNCKFGFWGP  NCTERRLLVR  RNIFDLSAPE  KDKFFAYLTL  AKHTISSDYV
151    IPIGTYGQMK  NGSTPMFNDI  NIYDLFVWMH  YYVSMDALLG  GSEIWRDIDF
201    AHEAPAFLPW  HRLFLLRWEQ  EIQKLTGDEN  FTIPYWDWRD  AEKCDICTDE
251    YMGGQHPTNP  NLLSPASFFS  SWQIVCSRLE  EYNSHQSLCN  GTPEGPLRRN
301    PGNHDKSRTP  RLPSSADVEF  CLSLTQYESG  SMDKAANFSF  RNTLEGFASP
351    LTGIADASQS  SMHNALHIYM  NGTMSQVQGS  ANDPIFLLHH  AFVDSIFEQW
401    LQRHRPLQEV  YPEANAPIGH  NRESYMVPFI  PLYRNGDFFI  SSKDLGYDYS
451    YLQDSDPDSF  QDYIKSYLEQ  ASRIWSWLLG  AAMVGAVLTA  LLAGLVSLLC
501    RHKRKQLPEE  KQPLLMEKED  YHSLYQSHL
```
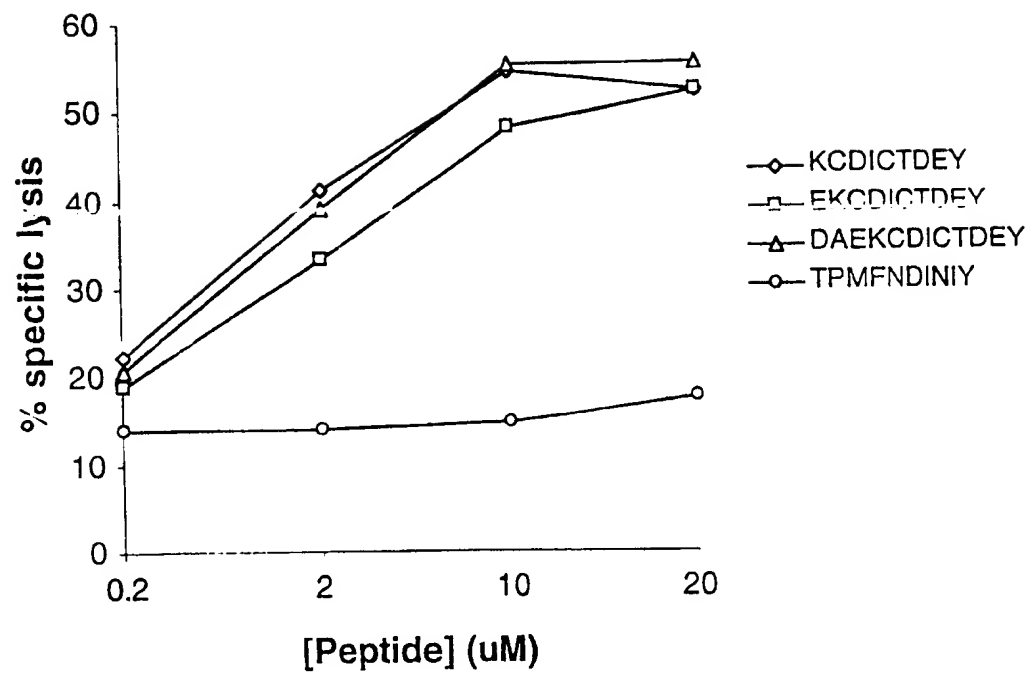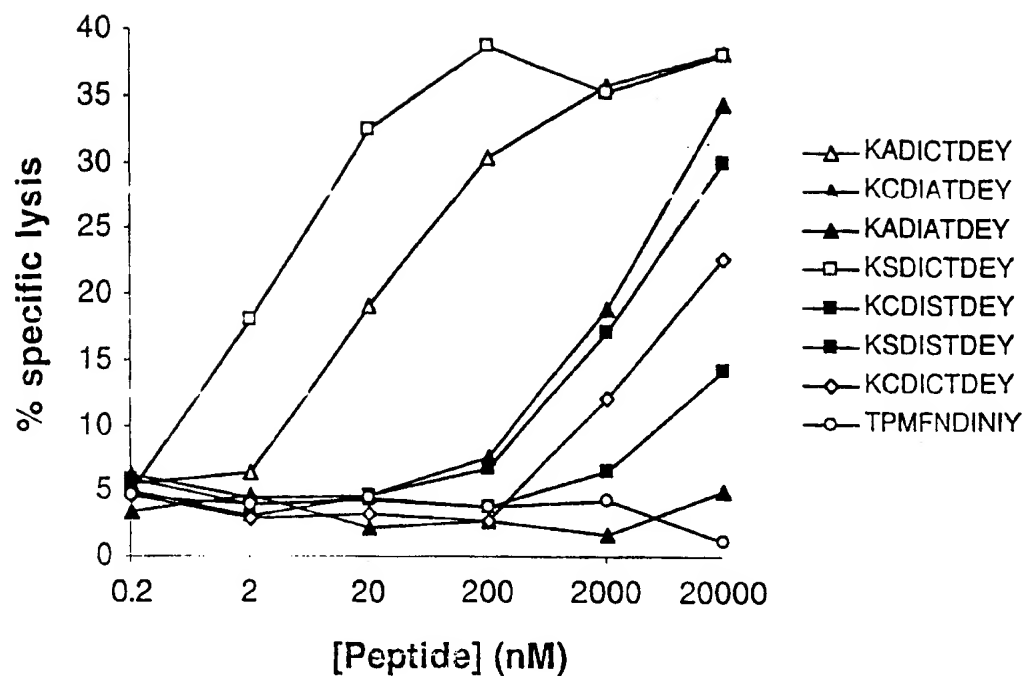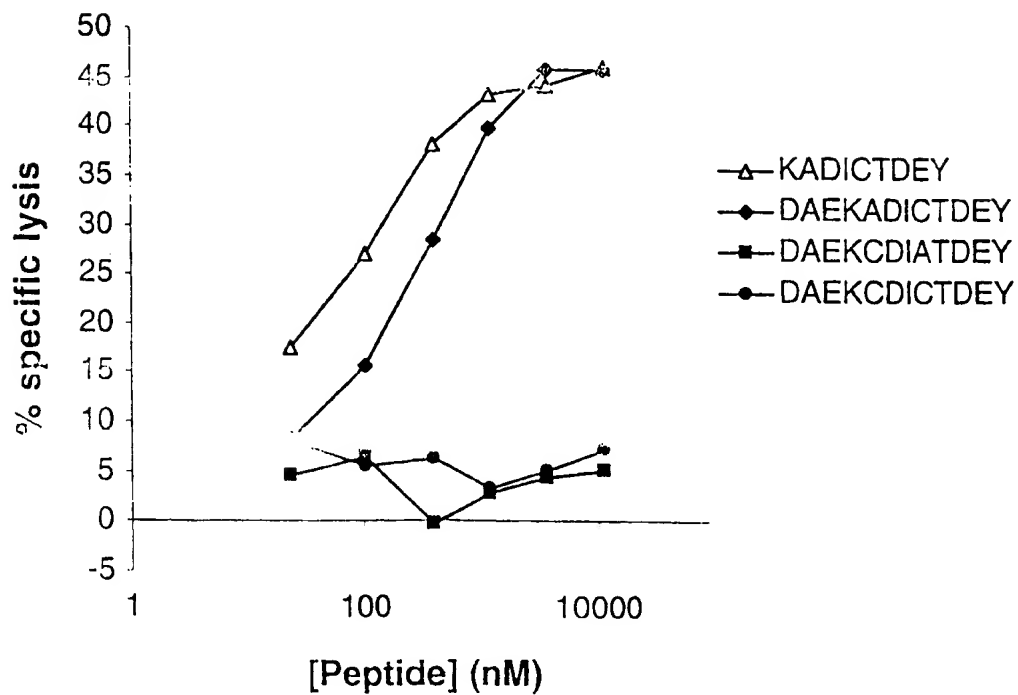
KCDICTDEY   represents residues 243 - 251 of the tyrosinase sequence

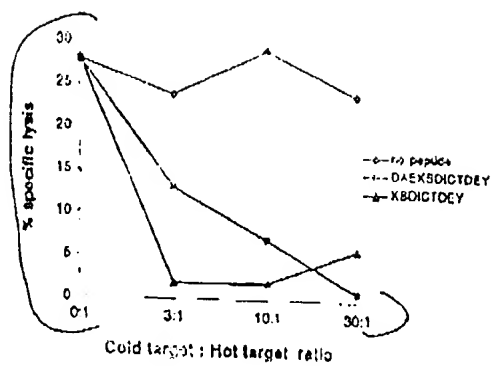|			
17/529 Residues are Cysteine	= 3.2 %	2/9 Residues of KCDICTDEY are Cysteine =	22 %
30/529 Residues are Aspartic acid	= 5.7 %	2/9 Residues of KCDICTDEY are Asp Acid =	22 %
27/529 Residues are Glutamic acid	= 5.1 %	3/9 Residues of KCDICTDEY are Glutamic acid =	11 %
17/529 Residues are Lysine	= 3.2 %	1/9 Residues of KCDICTDEY are Cysteine =	11 %
Total of C-D-E-K	= 17.2 %	=	67%

A

B

Figure 9